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One Hundred Eighty Day Subchronic Oral Toxicity Study of Pyridostigmine Bromide in Rats

(Volume 1 of 2)
(Part 1)

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DIVISION OF TOXICOLOGY

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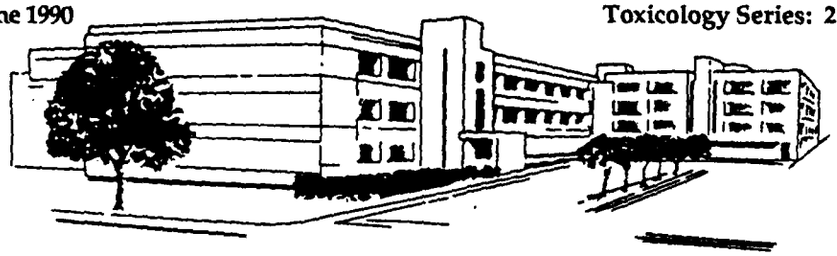

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One Hundred Eighty Day Subchronic Oral Toxicity Study of Pyridostigmine Bromide in Rats (Toxicology Series 253)--
Morgan *et al.*

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for William C Cole COL MC 15 June 90

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The 180-day subchronic oral toxicity of pyridostigmine bromide was evaluated in male Sprague-Dawley rats. Pyridostigmine was administered in the diet at dose levels of 0, 1, and 10 mg/kg/day daily, and 10 mg/kg/day 5 days per week for 180 days. Following the 180-day dosing period, subgroups of animals from the control, 10 mg/kg/day, and 10 mg/kg/day 5 days/week groups were subjected to a 30-day recovery period during which the test compound was not administered. The addition of pyridostigmine to the diet resulted in dose-related decreases in plasma cholinesterase and erythrocyte acetylcholinesterase activity ranging from 25% to 63% and from 21% to 49%, respectively. The only toxic sign associated with the decrease in cholinesterase activity was increased startle reflex which was observed with increased incidence among pyridostigmine-treated animals. Blood samples taken at necropsy for hematological and serum chemistry analyses exhibited no

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19 (cont.) significant ($p \leq 0.05$) abnormalities that could be attributed to pyridostigmine after 180 days of dosing. Increases in aspartate aminotransferase, lactate dehydrogenase, and creatine phosphokinase were observed at Day 210, 30 days after withdrawal of the test compound, but the changes could not be attributed to compound administration/withdrawal. No compound-related changes in food or water consumption or body weights were observed, and no morphologic signs of pyridostigmine-induced toxicity were detected during necropsy or subsequent microscopic examination of the tissues. These findings indicate that pyridostigmine bromide, when administered for 180 days to rats at doses that produce up to 63% inhibition of cholinesterase activity, produces little subchronic toxicity other than that attributable to cholinesterase inhibition.

ABSTRACT

The 180-day subchronic oral toxicity of pyridostigmine bromide was evaluated in male Sprague-Dawley rats. Pyridostigmine was administered in the diet at dose levels of 0, 1, and 10 mg/kg/day daily, and 10 mg/kg/day 5 days per week for 180 days. Following the 180-day dosing period, subgroups of animals from the control, 10 mg/kg/day, and 10 mg/kg/day 5 days/week groups were subjected to a 30-day recovery period during which the test compound was not administered. The addition of pyridostigmine to the diet resulted in dose-related decreases in plasma cholinesterase and erythrocyte acetylcholinesterase activity ranging from 25% to 63% and from 21% to 49%, respectively. The only toxic sign associated with the decrease in cholinesterase activity was increased startle reflex which was observed with increased incidence among pyridostigmine-treated animals. Blood samples taken at necropsy for hematological and serum chemistry analyses exhibited no significant ($p \leq 0.05$) abnormalities that could be attributed to pyridostigmine after 180 days of dosing. Increases in aspartate aminotransferase, lactate dehydrogenase, and creatine phosphokinase were observed at Day 210, 30 days after withdrawal of the test compound, but the changes could not be attributed to compound administration/withdrawal. No compound-related changes in food or water consumption or body weights were observed, and no morphologic signs of pyridostigmine-induced toxicity were detected during necropsy or subsequent microscopic examination of the tissues. These findings indicate that pyridostigmine bromide, when administered for 180 days to rats at doses that produce up to 63% inhibition of cholinesterase activity, produces little subchronic toxicity other than that attributable to cholinesterase inhibition.

Key Words: Subchronic Oral Toxicity, Pyridostigmine, Sprague-Dawley Rat, Acetylcholinesterase, Cholinesterase

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PREFACE

TYPE REPORT: 180-Day Subchronic Oral Toxicity GLP Study Report

TESTING FACILITY:
U.S. Army Medical Research and Development Command
Letterman Army Institute of Research
Presidio of San Francisco, CA 94129-6800

SPONSOR:
U.S. Army Medical Research and Development Command
U.S. Army Medical Materiel Development Activity
Fort Detrick, MD 21701-5009

PROJECT/WORK UNIT/APC: Pyridostigmine Projects/993/LLHO

GLP STUDY NUMBER: 86005

STUDY DIRECTOR: LTC Don W. Korte Jr., PhD, MSC
Diplomate, American Board of Toxicology

PRINCIPAL INVESTIGATOR:
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Diplomate, American College of Veterinary Preventive Medicine and American Board of Toxicology

CO-PRINCIPAL INVESTIGATORS:
Yvonne C. LeTellier, BS

SPC Joel B. Seewald, BS

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PATHOLOGIST: MAJ Charles B. Clifford, DVM, PhD, VC
Diplomate, ACVP

REPORT AND DATA MANAGEMENT:
A copy of the final report, study protocol, retired SOPs, raw data, analytical, stability, and purity data of the test compound, and an aliquot of the test compound will be retained in the LAIR Archives.

TEST SUBSTANCE: Pyridostigmine bromide

INCLUSIVE STUDY DATES: 21 Oct 86 - 2 Jun 87

OBJECTIVE:
The objective of this study was to determine the 180-day subchronic oral toxicity of pyridostigmine bromide in male Sprague-Dawley rats.

ACKNOWLEDGMENTS

SPC Theresa L. Polk, SGT Tammie Heineman, SPC Dean K. Magnuson, BS, SPC Scott L. Schwebe, Richard Katona, Gregory A. Rothhammer, Richard A. Spieler, Charlotte L. Gomez, and Obie Goodrich, Jr. provided research assistance and animal care; SPC Paul B. Simboli, BS, and SPC John R. G. Ryabik, BS, provided chemical preparation and analysis.

SIGNATURES OF PRINCIPAL SCIENTISTS INVOLVED IN THE STUDY

We, the undersigned, declare that GLP Study 86005 was performed under our supervision, according to the procedures described herein, and that this report is an accurate record of the results obtained.

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PAUL P. WARING, BS/DATE
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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

SGRD-ULZ-QA 29 May 1990

MEMORANDUM FOR RECORD

SUBJECT: GLP Compliance for GLP Study 86005

1. This is to certify that in relation to LAIR GLP Study 86005 the following inspections were made:

21 August 1986	-	Protocol review
11 March 1987	-	Terminal sacrifice, females
11 March 1985	-	Blood chemistry
18 March 1987	-	Micronucleus test
14 April 1987	-	Diet preparation
14 April 1987	-	Weigh rats/feeders, observations
05 May 1987	-	Observations and interim sacrifice, males
02 June 1987	-	Final sacrifice, males
02 June 1987	-	Final observations, weights

2. The institute report entitled "One Hundred Eighty-Day Subchronic Oral Toxicity Study of Pyridostigmine Bromide in Rats ," Toxicology Series 253, was audited on 29 May 1990.

Carolyn M. Lewis

CAROLYN M. LEWIS
Diplomate, American Board of Toxicology
Quality Assurance Auditor

TABLE OF CONTENTS

TABLE OF CONTENTS (cont.)

One Hundred Eighty-Day Subchronic Oral Toxicity Study of Pyridostigmine Bromide in Rats -- Morgan *et al.*

INTRODUCTION

Soman, the primary nerve agent utilized by threat forces, is refractory to the standard antidotal therapy, atropine and pralidoxime (2-PAM) chloride, which is currently fielded by the U.S. Army. Consequently, the U.S. Army Medical Research and Development Command (USAMRDC) has proposed a treatment regimen incorporating prophylaxis with a reversible cholinesterase inhibitor and, following nerve agent exposure, antidotal therapy with an oxime and an anticholinergic agent. The rationale for this approach is that the pretreatment will protect an adequate percentage (approximately 25%) of a soldier's cholinesterase from inhibition by a nerve agent without affecting his battlefield performance. Exposure to a nerve agent would irreversibly inhibit only the remaining cholinesterase. Antidotal therapy with atropine, an anticholinergic agent, and pralidoxime, an oxime, would accomplish two goals: the oxime would abate the inhibition induced by the reversible cholinesterase inhibitor prophylaxis, and the atropine will attenuate the excessive muscarinic response associated with cholinesterase inhibition. The immediate goal of the USAMRDC is to field a reversible cholinesterase inhibitor as the pretreatment component of a therapeutic regimen that would include antidotal therapy with 2-PAM chloride and atropine. A regimen incorporating pyridostigmine as a prophylactic agent, combined with standard atropine/2-PAM chloride therapy, has proven effective in reducing mortality of Rhesus monkeys following exposure to multilethal concentrations of soman (1).

Pyridostigmine is the drug of choice for the treatment of myasthenia gravis because of its relative lack of untoward effects in comparison with other anticholinesterases (2). This relative lack of clinical toxicity was reflected in animal studies conducted for Hoffman-La Roche by Pharmacology Research, Inc. The oral LD_{50} for pyridostigmine in rats was calculated as 87 mg/kg and was associated with signs of cholinergic and neuromuscular toxicity (3). Pyridostigmine was also fed to rats for 21 weeks, mixed in the feed at a maximum concentration of 0.064%, without producing significant toxicity or histological changes (4). These studies suggest that the only toxicological action of pyridostigmine is on cholinesterase activity, and that death

would occur acutely before morphological alterations could be observed. We have previously reported similar findings for pyridostigmine when administered in the feed to rats for 90 days (5). This study extends our earlier findings to 180 days of drug administration coupled with a 30-day wash-out period.

Objective of Study

The objective of this study was to determine the 180-day subchronic toxicity of pyridostigmine bromide in male Sprague-Dawley rats.

MATERIALS

Test Substance

Chemical name: P ostigmine bromide

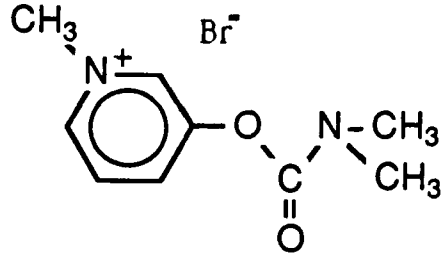
Chemical Abstracts Registry Number: 101-26-8

Lot Number: 590034

LAIR Code Number: TW71A

WRAIR Code Number: WR-250710AF

Chemical Structure:



Molecular Formula: $C_9H_{13}BrN_2O_2$

Other test substance information is presented in Appendix A.

Vehicle

The test compound was mixed into the feed (see Husbandry).

Animal Data

Sixty-nine male albino Sprague-Dawley rats (Charles River Laboratories, Inc., Portage, MI) were used in this study. Tail tattoos were used to identify each animal individually. Four animals were used for necropsy quality controls, and 10 animals were used as baseline controls. The body weights on receipt (21 Oct 1986) ranged from 101 g to 142 g. Additional animal data are presented in Appendix B.

Husbandry

The animals assigned to this study were housed individually in clear polycarbonate shoeboxes in drawer rack cages. Alpha-dri® (Shepherd Specialty Papers, Kalamazoo, MI), a cellulose fiber, was used as bedding. The shoeboxes and bedding were changed twice weekly. The diet, fed *ad libitum*, consisted of Certified Purina Rodent Chow® 5002 Meal Form (Ralston Purina, St. Louis, MO). Water was provided by 16-ounce water bottles with stoppers and sipper tubes. The temperature range maintained throughout this study was 20.0°C - 26.7°C with two brief decreases to a minimum of 13.3°C (22 Feb 87), and a relative humidity of 15-72%. Data collected on 22 February 1987 was unaffected by the transient temperature decrease. The photoperiod was 12 hours of light daily with a 1/2-hour dawn phase-in and a 1/2-hour dusk phaseout.

METHODS

This study was performed in accordance with LAIR SOP OP-STX-74, "Subchronic Oral Toxicity Testing in Rodents," (6) as presented in Appendix C, and FDA guidelines (7). The first 90 days of the study were performed in conjunction with a 90-day subchronic oral toxicity study of pyridostigmine in rats (5). Appendix D is a complete historical listing of study events.

Group Assignment/Acclimation

The animals were acclimated for 14 days from receipt to the onset of dosing. During the acclimation period, the animals were observed daily for signs of illness. Food and water consumption were measured during the second week of quarantine.

The study animals were assigned to groups, as presented in Table 1, using a random number generating program according to LAIR SOP OP-ISG-21 (8). Groups 4, 5, and 6 are

not listed in the table because those animals were part of the 90-day portion of GLP Study 86005, and were not included in the 180-day phase. The terms 3A and 7A are used to designate those animals of Groups 3 and 7, respectively, that were subjected to a 30-day recovery period after the 180-day dosing period, before sacrifice at day 210.

TABLE 1: Group Assignment

Group #	Dosing Scheme	Number of Animals	Necropsy Day
baseline	control	10	0
1	control	15	180 (10) 210 (5)
2	1.0 mg/kg/day, daily	10	180
3	10.0 mg/kg/day, daily	10	180
3A	10.0 mg/kg/day, daily for first 180 days followed by a 30-day recovery period	5	210
7	10.0 mg/kg/day, five days per week	10	180
7A	10.0 mg/kg/day five days per week for first 180 days followed by a 30-day recovery period	5	210

Dose Levels

The dose levels, 0, 1, and 10 mg/kg/day were selected on the basis of the results of a 14-day pilot study by Page and Emmerling (9) and the electron microscopy studies of Page (10). The 10 mg/kg/day, 5 days per week dosing regimen was selected to mimic a possible field expedient dosing regimen.

Compound and Diet Preparation

The pyridostigmine was received as a white crystalline material, 99.6% pure (by HPLC). All diets were prepared in accordance with LAIR SOP OP-STX-16 (11), as presented in

Appendix E. Preliminary studies indicated that pyridostigmine was stable in the feed for at least 8 days. New diets were prepared each week to compensate for changes in food consumption and body weight. Separate premixes consisting of appropriate concentrations of pyridostigmine and Rodent Chow were prepared for each final dietary concentration. On the day of the diet change, after the new diet concentrations had been calculated, the appropriate amounts of premix and meal were blended together using a Patterson-Kelley Twin-Shell® Blender (Patterson-Kelley Co., Division of Harsco Corp., East Stroudsburg, PA) for at least 15 minutes. Pyridostigmine was mixed into the feed at a level that, based on the feed consumption of the previous week and the animal's weight, would provide the desired dose (mg/kg) on a daily basis. With the exception of 11% of the diet mixture samples that were within 10-15% of target concentrations, all diet mixes were within 10% of target concentration and were adequately homogeneous. Additional mixing data and analyses are presented in Appendix F.

Test Procedures

Food and water consumption were measured on a weekly basis. Individual feed jars were weighed at the beginning and end of each week. The feed was sifted using a 10-mesh sieve to remove bedding and feces prior to the final weighing. If there were signs of spillage in the bedding, the bedding was also sifted and the feed obtained was returned to the jar prior to weighing. Records for water bottles with obvious spillage were flagged, and the weights were omitted. Recordkeeping initiated during the final week of quarantine provided the baseline consumption data to calculate the first week's diet mixture.

Early on the day of diet change, the animals were weighed and observed, and their water bottles and feeders were weighed. These data were collected on a Beckman TOXSYS® data collection terminal. The Beckman Diet Computation Subsystem was used for the calculations. After the new diet was mixed, the feeders and water bottles were filled, weighed, and returned to the cages.

Observations were performed twice daily throughout the 210-day test period. During the morning observations, the animals were observed undisturbed in their cages, outside of their cages, and after return to their cages. All findings were recorded. A second "walk through" observation was performed in the afternoon, and only significant observations were recorded. Body weights were recorded weekly and on the day of sacrifice.

All animals were subjected to a complete necropsy following exsanguination under sodium pentobarbital anesthesia on Day 180, or Day 210 after a 30-day recovery period. Under anesthesia, blood was collected from the right ventricle for serum chemistry, hematology, plasma cholinesterase (ChE), and erythrocyte acetylcholinesterase (AChE) activity measurements. Samples for ChE and AChE activity measurements were prepared and analyzed using a Technicon Auto-Analyzer II System in accordance with LAIR SOP OP-ACH-83 (12), Appendix G. The following tissues were examined microscopically for all groups: diaphragm, *extensor digitorum longus* muscle, soleus muscle, kidney, liver, lungs, adrenal glands, aorta, femur, brain, sternum, bone marrow, cecum, colon, duodenum, lacrimal gland, esophagus, eyes with optic nerve and extraocular muscle, heart, ileum, jejunum, mesenteric lymph node, nose/turbinates, pancreas, pituitary, parathyroid, spinal cord, vertebrae, skin, salivary gland, skeletal muscle, sciatic nerve, spleen, stomach, thyroid, thymus, trachea, urinary bladder, accessory sex organs, testes, epididymus and all gross lesions.

Statistical Analysis

Data for body weights, food consumption, water consumption, serum chemistry, hematology, and cholinesterase activity were analyzed statistically with programs available on BMDP software (13). The equality of the variances of the groups was tested using the Levene's Test. If the variances were equal, the vehicle control group and the dose groups were compared by the standard one-way analysis of variance (ANOVA). Otherwise, the Welch one-way ANOVA, which is not based on the assumption that the variances are equal, was performed. If the F-statistic was significant in either case, the Dunnett's *t* test was performed to determine whether or not the vehicle control group was significantly different from any of the dose groups. The food consumption data for Group 7, 10 mg/kg/day, 5 days per week, was recorded on a 5-day basis for the period during which the group was receiving the test compound (weeks 1-26). This data was converted to a 7-day basis by multiplying by a factor of 7/5 to facilitate statistical comparison of food consumption data among the study groups. Percent inhibition of cholinesterase activities were calculated as [(mean baseline activity - normalized mean treated activity) ÷ mean baseline activity] x 100%. The normalized mean treated activity was calculated as (mean baseline activity ÷ mean control group activity) x mean treatment group activity. Statistical analyses for organ weights were done on the Xybion software program using the standard one-way ANOVA. The homogeneity of the groups was

tested by the Bartlett's test. If the groups were found to be non-homogeneous, then a modified *t* test was performed instead of the Dunnett's *t* test. The incidence of microscopic lesions for each test group was compared to the control group using the Kolmogorov-Smirnov two-tailed test. The 0.05 level of significance was used for all tests.

Changes/Deviations

This study was accomplished according to the protocol and applicable amendments with the following exceptions: During the first week of quarantine, difficulties in adapting to the watering system resulted in slow growth for a number of study animals. The TOXSYS® Animal Allocation Program could not provide satisfactory randomization due to the increased variation in body weights. Therefore, the study animals were assigned to dose groups using a random number generating program. Clinical signs from 14-21 May 87 were inadvertently not recorded. Due to constraints of the XYBION program, Groups 3A and 7A are designated as Groups 8 and 9, respectively, in the pathology report and XYBION generated pathology tables. These deviations did not affect the outcome of this study.

Storage of Raw Data and Final Report

A copy of the final report, study protocols, raw data, retired SOPs, and an aliquot of the test compound will be retained in the LAIR Archives.

RESULTS

Food and Water Consumption

Mean daily consumption of pyridostigmine is presented in Table 2. Mean weekly food and water consumption data are presented in Tables 3 and 4, respectively. Individual pyridostigmine, food, and water consumption data are presented in Appendices H, I, and J, respectively. Individual pyridostigmine consumption was calculated based on the mean of each animal's body weights taken at the beginning and end of each week, the individual food consumption of the animal for that week, and the pyridostigmine concentration as determined by analysis of the feed mixtures. The mean daily pyridostigmine consumption taken for the entire 26-week period of test compound administration ranged from 95.8-97.4% of the target doses.

Compared to the controls, statistically significant decreases in food consumption were observed for Group 7 during weeks 3 and 21 of the study period. The decreases were isolated occurrences without clinical significance. No significant differences from controls were observed in the water consumption of pyridostigmine-treated animals during the study period.

Body Weights

Individual body weight data are presented in Appendix K. The group mean body weight data are presented in Table 5. No significant differences from controls were observed in the body weights of pyridostigmine-treated animals during the study period.

Clinical Observations

A summary of clinical observations is presented in Table 6. Individual animal histories are presented in Appendix L. The clinical signs observed were grouped into behavioral, reflexive, respiratory, skin/fur, ocular, gastrointestinal, and general categories. With the exception of the reflexive and gastrointestinal signs, all categories were observed with relatively equal or decreased incidence among the treated animals compared to the controls. No deaths occurred during the study.

The most frequently observed signs were of the behavioral category (40 of 40 pyridostigmine-treated animals). These signs included irritability, aggression, inactivity, chewing, jumping, and vocalization. With the exception of a slight increase in inactivity for Group 7, all behavioral signs were observed with relatively equal incidence among the treated and control groups. Irritability and aggression were the most prominent behavioral signs, while inactivity, chewing, jumping, and vocalization occurred sporadically.

The only reflexive sign observed, increased startle reflex (18 of 40), appeared to be dose-related, occurring most frequently in the high-dose groups.

Respiratory signs characterized by stains or material around the nose (36 of 40) were observed with relatively equal distribution among the treated and control groups with nearly all animals exhibiting the signs.

Abnormalities of the skin/fur occurred with decreased incidence in the pyridostigmine-treated animals (20 of 40)

compared to the controls. The difference was attributable to increased alopecia and stains or material on various parts of the body for the control animals. Other skin/fur signs were observed with relatively equal distribution among treated and control groups.

The only ocular sign observed in pyridostigmine-treated animals (3 of 40) was stain or material around the eyes. This sign was observed with increased incidence in the control group. Conjunctivitis was observed only in the control group.

Gastrointestinal signs observed (6 of 40) included perianal stain/feces, stains around the mouth, and diarrhea. The occurrence of gastrointestinal signs was sporadic and relatively equal among the pyridostigmine-treated and control groups.

The general sign, dehydration, was observed as one isolated case in Group 7.

Signs observed during the 30-day recovery period included irritability, aggression, stains or material around the nose, alopecia, stains or material on various parts of the body, and scabs. All were observed with relatively equal distribution among the controls and Groups 3A and 7A.

Serum Chemistry

Individual serum chemistry values are presented in Appendix M. A summary of serum chemistry data is presented in Table 7. At Day 180, the only statistically significant variation from control serum chemistry measurements was a slight increase in calcium (CAL) observed for Group 7. The mean CAL level for Group 7, however, remained within normal limits determined by Day 0 baseline measurements. At Day 210, statistically significant increases from control values were observed for aspartate aminotransferase (AST, Group 7A), lactate dehydrogenase (LDH, Group 3A and 7A), and creatine phosphokinase (CK, Group 3A and 7A). At this time, a significant decrease was observed for chloride (CL, Group 7A), but the CL value remained within clinically acceptable normal limits.

Hematology

Individual hematology data are presented in Appendix N. Group mean summary data are presented in Table 8. No statistically significant variations from control hematology measurements were observed during the study period.

Cholinesterase Activity

Individual plasma cholinesterase (ChE) and erythrocyte acetylcholinesterase (AChE) activities are presented in Appendix M. Group mean ChE and AChE activity data are presented in Table 7. Percent inhibition calculations are presented in Table 9. At Day 180, ChE and AChE activity levels exhibited statistically significant, dose-related decreases compared to control values. At Day 210, following a 30-day recovery period, the mean ChE activity levels for Groups 3A and 7A, and the mean AChE level for Group 3A returned to values comparable to those of the control group. The AChE activity of Group 7A, however, remained significantly depressed compared to the controls. At the conclusion of the 180-day dosing period, percent inhibition ranged from ~25% to 63% (ChE) and ~21% to 49% (AChE).

Necropsy Findings

No morphologic evidence of pyridostigmine induced toxicity was observed. All gross lesions were considered to be incidental findings commonly observed in Sprague-Dawley rats. Microscopic lesions observed with significantly increased incidence in pyridostigmine-treated groups compared to the controls included chronic, multifocal hepatic inflammation (Group 3) and brown pigment, probably hemosiderin, within splenic macrophages (Group 7A). However, the microscopic lesions were also observed in the controls and were considered to be incidental findings unrelated to treatment. The pathology report is presented in Appendix O.

DISCUSSION

After 180 days of test compound administration, doses of pyridostigmine that produced up to 63% cholinesterase inhibition in plasma and 49% acetylcholinesterase inhibition in erythrocytes did not have toxic effects other than those attributable to cholinergic stimulation. No mortalities occurred, and no consistent treatment-related changes attributable to pyridostigmine administration were observed in food consumption, water consumption, body weights, serum chemistry, or hematology values during the 180-day dosing period. In addition, at necropsy and upon microscopic examination of tissues, no lesions were noted which could be attributed to pyridostigmine administration.

Following the 30-day recovery period, however, statistically significant increases in lactate dehydrogenase

(LDH) and creatine phosphokinase (CK) were observed for Groups 3A and 7A (10 mg/kg/day pyridostigmine for 7 and 5 days per week, respectively) compared to the controls. The mean aspartate aminotransferase (AST) level for Group 7A was also significantly elevated compared to the controls at Day 210. Increases in LDH, CK, and AST have all been associated with myopathies (14). However, morphologic evidence of increased incidence of myopathy/muscle damage was not observed grossly or upon microscopic examination of tissues. Furthermore, the LDH, CK, and AST values were extremely variable throughout the study period for baseline and control group animals, and were beyond generally accepted normal ranges for the rat (15). The variation in the enzyme activities within each group across time, and intergroup variation may have been due to factors such as sample hemolysis and cardiac muscle damage induced by sample collection technique (cardiac puncture) rather than toxic effects of the test compound. In addition, these enzymes are indicators of acute damage, and the increases occurred 30 days after pyridostigmine had been removed from the feed.

The clinical signs of toxicity observed were consistent with cholinergic stimulation following cholinesterase inhibition (16,17). Increased startle reflex may have been due to peripheral nicotinic effects or possibly stimulation of the central nervous system as has been observed with other anti-ChE agents. This would be consistent with CNS signs such as confusion, ataxia, slurred speech, and loss of reflexes, which have been observed in humans exposed to anti-ChE agents (16).

Pyridostigmine reduced plasma cholinesterase and erythrocyte acetylcholinesterase significantly ($p \leq 0.05$). The failure of the red cell acetylcholinesterase of Group 7A to return to baseline levels 30 days after the cessation of dosing could be attributed to several factors, the most important being that the large sample volume required for the cholinesterase determination precluded each animal from serving as its own baseline. Consequently, the percent cholinesterase inhibition for a particular treatment required normalizing the mean cholinesterase activity for baseline control animals obtained on Day 0 and the mean activity for the Day 180 or Day 210 concurrent control group. Normal interanimal variations in resting cholinesterase levels, therefore, could account for the low value observed at Day 210. Other factors contributing to the depressed AChE activity may have been the small number of animals (n=5/group) for the Day 210 determinations and differences in age of the animals when activity was determined (7-8 weeks at Day 0, 33-34 weeks at Day 180, and 37-38 weeks at Day 210).

CONCLUSION

Administration of pyridostigmine at doses of 1 to 10 mg/kg/day for 180 days did not cause any appreciable toxicologic effects. Increases in aspartate aminotransferase, lactate dehydrogenase, and creatine phosphokinase were observed 30 days following withdrawal of the test compound, but could not be attributed to compound administration. Clinical signs of cholinergic stimulation due to subchronic inhibition of plasma ChE and erythrocyte AChE were present but were considered to be mild and nondebilitating.

TABLE 2: Daily Consumption of Pyridostigmine

Week	n	Control (mg/kg/day)	n	Group 2 (mg/kg/day)
1	15	0.00* ± 0.00	10	1.18 ± 0.03
2	15	0.00 ± 0.00	10	1.07 ± 0.02
3	15	0.00 ± 0.00	10	0.92 ± 0.01
4	15	0.00 ± 0.00	10	0.95 ± 0.01
5	15	0.00 ± 0.00	10	0.90 ± 0.01
6	15	0.00 ± 0.00	10	1.05 ± 0.01
7	15	0.00 ± 0.00	10	0.99 ± 0.04
8	15	0.00 ± 0.00	10	0.88 ± 0.02
9	15	0.00 ± 0.00	10	0.89 ± 0.01
10	15	0.00 ± 0.00	10	0.91 ± 0.01
11	15	0.00 ± 0.00	10	0.92 ± 0.01
12	15	0.00 ± 0.00	10	0.81 ± 0.04
13	15	0.00 ± 0.00	10	1.04 ± 0.02
14	15	0.00 ± 0.00	10	0.92 ± 0.01
15	15	0.00 ± 0.00	10	0.94 ± 0.01
16	15	0.00 ± 0.00	10	0.95 ± 0.02
17	15	0.00 ± 0.00	10	0.92 ± 0.02
18	15	0.00 ± 0.00	10	0.95 ± 0.02
19	15	0.00 ± 0.00	10	0.95 ± 0.03
20	15	0.00 ± 0.00	10	0.96 ± 0.02
21	15	0.00 ± 0.00	10	1.06 ± 0.02
22	15	0.00 ± 0.00	10	0.93 ± 0.03
23	15	0.00 ± 0.00	10	1.01 ± 0.03
24	15	0.00 ± 0.00	9	1.03 ± 0.03
25	15	0.00 ± 0.00	9	1.03 ± 0.06
26	15	0.00 ± 0.00	10	1.16 ± 0.04
27	5	0.00 ± 0.00		
28	5	0.00 ± 0.00		
29	5	0.00 ± 0.00		
30	5	0.00 ± 0.00		

* Data are presented as the mean ± the standard error.

TABLE 2 (cont.): Daily Consumption of Pyridostigmine

Week	n	Group 3/3A (mg/kg/day)	n	Group 7/7A (mg/kg/day)
1	15	9.30* ± 0.28	15	9.76 ± 0.27
2	15	9.05 ± 0.28	15	8.63 ± 0.17
3	15	8.78 ± 0.22	15	7.44 ± 0.16
4	15	9.52 ± 0.22	15	8.40 ± 0.12
5	15	8.43 ± 0.16	15	8.82 ± 0.15
6	15	10.31 ± 0.16	15	9.75 ± 0.18
7	15	10.42 ± 0.14	15	10.72 ± 0.15
8	15	10.02 ± 0.18	15	9.49 ± 0.14
9	15	9.35 ± 0.15	15	9.03 ± 0.19
10	15	9.27 ± 0.21	15	9.05 ± 0.14
11	15	9.54 ± 0.18	15	8.91 ± 0.21
12	15	9.32 ± 0.10	15	9.20 ± 0.23
13	15	10.10 ± 0.17	15	10.81 ± 0.20
14	14	8.51 ± 0.48	15	10.02 ± 0.14
15	15	9.68 ± 0.12	15	9.14 ± 0.16
16	14	9.47 ± 0.12	15	10.13 ± 0.18
17	15	10.48 ± 0.21	15	9.91 ± 0.24
18	15	9.92 ± 0.13	15	10.61 ± 0.24
19	15	9.29 ± 0.19	15	10.16 ± 0.17
20	5	10.52 ± 0.22	15	9.99 ± 0.18
21	4	9.75 ± 0.17	15	10.18 ± 0.24
22	15	9.99 ± 0.13	15	10.40 ± 0.17
23	14	8.66 ± 0.43	15	10.17 ± 0.28
24	11	9.43 ± 0.22	14	10.65 ± 0.19
25	15	10.34 ± 0.20	15	10.08 ± 0.21
26	15	9.67 ± 0.14	15	9.18 ± 0.18
27	5	0.00 ± 0.00	5	0.00 ± 0.00
28	5	0.00 ± 0.00	5	0.00 ± 0.00
29	5	0.00 ± 0.00	5	0.00 ± 0.00
30	5	0.00 ± 0.00	5	0.00 ± 0.00

* Data are presented as the mean ± the standard error.

TABLE 3: Food Consumption Summary

Week	n	Control (g/week)	n	Group 2 (g/week)
QW2	15	140.1* ± 7.8	10	141.1 ± 6.8
1	15	157.1 ± 4.0	10	163.5 ± 7.9
2	15	172.0 ± 4.6	10	171.7 ± 5.7
3	15	170.4 ± 3.3	10	174.8 ± 6.0
4	15	174.3 ± 3.7	10	180.3 ± 6.3
5	15	176.4 ± 3.9	10	176.8 ± 5.9
6	14	174.0 ± 4.8	10	176.9 ± 6.6
7	15	177.7 ± 4.7	10	184.6 ± 5.5
8	15	177.9 ± 4.7	10	184.6 ± 4.6
9	15	171.9 ± 8.5	10	183.9 ± 4.6
10	15	178.5 ± 6.2	10	185.8 ± 6.3
11	13	164.4 ± 7.8	10	181.1 ± 6.1
12	15	189.1 ±13.2	10	165.7 ± 8.0
13	15	179.2 ± 7.2	10	180.3 ± 5.6
14	15	177.1 ± 6.5	10	179.5 ± 4.8
15	15	172.8 ± 6.0	10	175.9 ± 5.6
16	15	173.1 ± 5.4	10	178.4 ± 5.3
17	15	166.3 ± 5.4	10	180.0 ± 5.1
18	15	170.3 ± 4.1	10	180.5 ± 5.2
19	15	166.6 ± 3.7	10	174.9 ± 5.1
20	15	181.3 ± 6.3	10	184.2 ± 5.6
21	15	184.0 ± 5.7	10	196.1 ± 6.2
22	15	172.5 ± 4.0	10	179.2 ± 4.7
23	15	177.1 ± 4.9	10	182.9 ± 4.2
24	15	175.5 ± 5.3	9	189.9 ± 4.9
25	15	176.1 ± 5.1	9	176.9 ±12.2
26	15	180.4 ± 4.7	10	182.3 ± 4.2
27	5	226.0 ±58.6		
28	5	146.4 ±13.7		
29	5	145.8 ±11.0		
30	5	145.4 ±12.2		

* Data are presented as the mean ± the standard error.

TABLE 3 (cont.): Food Consumption Summary

Week	n	Group 3/3A (g/week)	n	Group 7/7A (g/week)
QW2	15	140.8* ± 2.7	15	143.9 ± 4.5
1	15	158.5 ± 3.8	15	163.4 ± 4.1
2	15	164.9 ± 3.8	15	171.3 ± 3.6
3	15	167.3 ± 3.8	15	154.7@ ± 3.9
4	15	170.3 ± 3.8	15	174.4 ± 4.3
5	15	172.3 ± 3.8	15	162.9 ± 4.6
6	15	176.1 ± 3.6	15	175.4 ± 3.2
7	15	173.1 ± 3.3	15	181.3 ± 3.8
8	15	180.9 ± 3.8	15	183.7 ± 4.3
9	15	176.6 ± 4.7	15	174.4 ± 3.5
10	15	179.1 ± 5.2	15	178.3 ± 3.5
11	15	175.7 ± 4.5	15	175.5 ± 5.6
12	15	168.8 ± 3.0	15	169.8 ± 5.0
13	15	173.7 ± 3.8	15	178.3 ± 4.9
14	14	159.8 ± 9.6	15	183.3 ± 5.3
15	15	172.1 ± 4.6	15	181.1 ± 5.1
16	14	172.2 ± 4.3	15	169.3 ± 5.3
17	15	170.6 ± 4.2	15	162.1 ± 5.1
18	15	171.4 ± 3.8	15	173.8 ± 6.1
19	15	168.5 ± 4.7	15	173.7 ± 3.7
20	15	178.7 ± 5.1	15	175.4 ± 4.9
21	14	175.8 ± 3.9	15	166.9@ ± 3.9
22	15	173.4 ± 3.7	15	170.2 ± 4.2
23	14	169.6 ± 8.8	15	171.9 ± 6.4
24	11	171.5 ± 3.6	14	179.6 ± 4.1
25	15	179.4 ± 4.2	15	173.9 ± 6.0
26	15	172.0 ± 4.8	15	167.9 ± 4.9
27	5	151.4 ± 7.9	5	156.0 ± 7.8
28	5	145.6 ± 6.2	5	136.8 ±22.5
29	5	139.4 ± 7.1	5	146.4 ± 9.2
30	5	136.6 ± 4.4	5	137.0 ± 3.8

* Data are presented as the mean ± the standard error.
@ Significant difference from controls at $p \leq 0.05$.

TABLE 4: Water Consumption Summary

Week	n	Control (ml/week)	n	Group 2 (ml/week)
QW2	15	214.5* ± 9.3	9	217.1 ± 14.0
1	15	238.5 ± 9.0	10	257.9 ± 15.6
2	15	248.1 ± 8.7	10	263.5 ± 17.0
3	15	257.7 ± 8.7	10	271.7 ± 12.5
4	15	265.7 ± 9.9	10	290.0 ± 24.6
5	15	260.9 ± 11.7	10	262.6 ± 14.1
6	15	256.1 ± 12.6	10	264.0 ± 14.5
7	15	254.0 ± 13.3	10	283.3 ± 19.3
8	15	256.6 ± 11.9	10	266.7 ± 15.1
9	15	243.1 ± 12.4	10	254.6 ± 13.4
10	15	244.9 ± 13.6	10	253.9 ± 15.1
11	15	237.5 ± 14.9	10	258.5 ± 17.1
12	15	245.2 ± 14.1	10	255.6 ± 14.7
13	15	226.5 ± 12.1	10	252.3 ± 15.2
14	15	235.5 ± 14.0	10	240.5 ± 12.1
15	15	226.7 ± 11.8	10	251.3 ± 16.9
16	15	214.7 ± 11.9	10	236.1 ± 12.6
17	15	220.9 ± 13.0	10	245.3 ± 12.8
18	15	220.3 ± 12.0	10	266.0 ± 15.3
19	15	217.9 ± 11.7	10	253.5 ± 16.6
20	15	250.9 ± 14.1	10	267.3 ± 13.4
21	15	245.2 ± 15.1	10	264.5 ± 17.5
22	15	229.3 ± 12.9	10	246.3 ± 11.1
23	15	240.1 ± 14.1	10	249.8 ± 14.1
24	15	245.6 ± 14.5	10	260.2 ± 12.6
25	15	238.9 ± 13.6	9	266.7 ± 17.0
26	15	221.2 ± 13.3	10	239.5 ± 12.5
27	4	250.8 ± 72.0		
28	5	225.0 ± 38.7		
29	5	222.0 ± 31.4		
30	5	216.8 ± 24.1		

* Data are presented as the mean ± the standard error.

TABLE 4 (cont.): Water Consumption Summary

Week	n	Group 3/3A (ml/week)	n	Group 7/7A (ml/week)
QW2	15	212.7* ± 6.0	15	216.3 ± 13.0
1	15	247.0 ± 8.0	15	255.7 ± 15.3
2	14	262.2 ± 9.4	15	277.6 ± 16.2
3	15	263.4 ± 9.7	15	276.3 ± 18.1
4	15	268.8 ± 11.0	15	280.0 ± 19.2
5	15	247.6 ± 11.8	15	246.3 ± 10.4
6	15	261.3 ± 12.0	15	274.1 ± 18.1
7	15	252.9 ± 13.0	15	276.8 ± 19.2
8	15	249.1 ± 12.6	14	279.6 ± 20.6
9	15	246.3 ± 14.6	15	260.9 ± 20.3
10	15	242.3 ± 12.2	14	260.3 ± 18.1
11	15	246.9 ± 11.9	13	262.5 ± 19.5
12	15	239.9 ± 12.8	15	270.2 ± 23.2
13	15	229.5 ± 12.1	14	246.6 ± 16.3
14	15	215.8 ± 11.2	14	250.8 ± 16.1
15	15	221.7 ± 10.6	15	252.1 ± 18.1
16	14	216.9 ± 10.6	15	244.0 ± 18.8
17	15	226.9 ± 11.8	14	239.7 ± 16.7
18	15	228.7 ± 11.6	14	239.8 ± 14.7
19	15	233.7 ± 13.0	15	256.6 ± 17.7
20	15	233.8 ± 12.1	15	266.5 ± 20.3
21	15	244.5 ± 11.9	15	253.7 ± 19.0
22	15	232.4 ± 11.5	15	242.5 ± 18.9
23	15	234.3 ± 10.9	15	249.0 ± 17.4
24	15	233.1 ± 11.1	15	249.1 ± 18.9
25	15	235.8 ± 11.7	15	243.7 ± 19.9
26	15	226.7 ± 12.7	15	238.5 ± 21.0
27	5	194.2 ± 20.1	5	250.8 ± 24.8
28	5	178.2 ± 18.6	5	297.4 ± 42.2
29	5	204.8 ± 21.2	5	240.8 ± 22.0
30	5	206.6 ± 23.2	5	247.2 ± 17.9

* Data are presented as the mean ± the standard error.

TABLE 5: Body Weight Summary

Week	n	Control (g)	n	Group 2 (g)
RPT	15	116.0* ± 2.0	10	118.5 ± 4.4
ALLOC	15	148.6 ± 9.8	10	137.4 ± 15.3
QW1	15	160.7 ± 8.7	10	144.3 ± 16.1
QW2	15	223.3 ± 7.8	10	213.9 ± 13.6
1	15	280.8 ± 7.2	10	274.3 ± 14.1
2	15	321.2 ± 7.1	10	321.7 ± 13.2
3	15	360.9 ± 7.4	10	356.2 ± 16.3
4	15	391.3 ± 7.9	10	395.4 ± 14.6
5	15	430.2 ± 11.6	10	423.1 ± 15.2
6	15	457.8 ± 10.3	10	448.3 ± 16.2
7	15	473.2 ± 10.6	10	471.7 ± 16.7
8	15	490.6 ± 11.6	10	490.3 ± 16.7
9	15	504.7 ± 11.8	10	508.4 ± 17.6
10	15	518.9 ± 14.2	10	523.1 ± 18.8
11	15	534.2 ± 13.4	10	540.8 ± 19.5
12	15	546.7 ± 14.7	10	554.9 ± 20.5
13	15	558.3 ± 15.2	10	566.9 ± 21.4
14	15	567.4 ± 16.5	10	581.1 ± 22.0
15	15	579.9 ± 17.2	10	586.4 ± 22.8
16	15	590.3 ± 17.6	10	600.3 ± 24.4
17	15	598.7 ± 18.2	10	614.3 ± 25.0
18	15	603.3 ± 17.9	10	622.2 ± 26.3
19	15	607.3 ± 17.9	10	619.1 ± 26.8
20	15	617.5 ± 18.7	10	632.6 ± 27.4
21	15	624.5 ± 18.6	10	640.9 ± 28.0
22	15	634.6 ± 18.5	10	652.5 ± 27.9
23	15	636.6 ± 19.7	10	659.6 ± 27.5
24	15	645.9 ± 19.4	10	671.5 ± 27.6
25	15	653.6 ± 19.9	10	670.3 ± 31.0
26	15	662.1 ± 20.1	10	680.2 ± 30.1
27	10	654.8 ± 27.9	10	686.5 ± 30.2
28	5	663.8 ± 55.6		
29	5	674.4 ± 56.9		
30	5	676.6 ± 57.7		

* Data are presented as the mean ± the standard error.

TABLE 5 (cont.): Body Weight Summary

Week	Group 3/3A n	Group 3/3A (g)	Group 7/7A n	Group 7/7A (g)
RPT	15	113.4* ± 2.2	15	119.7 ± 3.0
ALLOC	15	142.6 ± 10.1	15	139.9 ± 10.9
QW1	15	149.6 ± 10.6	15	155.9 ± 9.4
QW2	15	214.5 ± 9.1	15	220.8 ± 8.5
1	15	268.3 ± 8.3	15	259.4 ± 7.9
2	15	309.2 ± 6.4	15	320.5 ± 7.6
3	15	347.1 ± 7.4	15	352.7 ± 8.0
4	15	378.7 ± 7.0	15	383.1 ± 8.3
5	15	407.4 ± 7.4	15	410.6 ± 8.4
6	15	430.7 ± 7.4	15	435.9 ± 8.9
7	15	450.7 ± 7.7	15	459.1 ± 10.0
8	15	470.2 ± 8.1	15	477.5 ± 10.4
9	15	486.1 ± 9.2	15	489.7 ± 10.3
10	15	512.1 ± 10.7	15	510.9 ± 9.6
11	15	514.2 ± 9.4	15	521.3 ± 9.6
12	15	530.4 ± 9.7	15	532.8 ± 11.1
13	15	542.5 ± 10.3	15	546.5 ± 11.2
14	15	550.5 ± 9.9	15	562.3 ± 12.3
15	15	563.5 ± 11.2	15	565.1 ± 13.3
16	15	573.5 ± 11.8	15	578.1 ± 13.8
17	15	583.5 ± 11.5	15	584.6 ± 14.6
18	15	591.9 ± 12.0	15	593.2 ± 14.1
19	15	595.5 ± 12.5	15	596.9 ± 14.4
20	15	604.2 ± 12.9	15	607.7 ± 14.5
21	15	614.1 ± 13.1	15	610.7 ± 14.7
22	15	623.1 ± 13.7	15	617.0 ± 14.6
23	15	628.3 ± 13.7	15	625.8 ± 15.0
24	15	634.3 ± 13.6	15	626.7 ± 14.5
25	15	644.4 ± 14.3	15	637.8 ± 15.4
26	15	644.5 ± 14.9	15	640.3 ± 16.0
27	15	652.9 ± 14.4	15	633.8 ± 30.3
28	5	645.0 ± 30.7	5	621.8 ± 41.4
29	5	646.8 ± 31.9	5	635.6 ± 34.2
30	5	649.2 ± 30.6	5	637.6 ± 32.0

* Data are presented as the mean ± the standard error.

TABLE 6: Clinical Observations Summary*

Group	Control	2	3/3A	7/7A
n	15	10	15	15
Observation				
	Males			
BEHAVIORAL	14	10	15	15
IRRITABLE	14	10	15	15
AGGRESSIVE	11	7	11	10
INACTIVE	1	-	-	3
CHEWING	-	1	-	1
JUMPING	-	-	1	-
VOCALIZATION	1	-	-	-
REFLEXIVE	4	3	7	8
INCR. STARTLE REFLEX	4	3	7	8
RESPIRATORY	15	9	13	14
STAIN/MATERIAL NOSE	15	9	13	14
SKIN/FUR	10	6	7	7
ROUGH COAT	6	4	6	4
ALOPECIA	5	3	2	4
STAIN/MATERIAL LEG, BACK, NECK, HEAD, EAR, ABDOMEN, FOOT	5	2	2	1
SCAB	1	1	1	-
SWOLLEN, RED FOOT	-	-	1	-
OCULAR	5	3	-	-
STAIN/MATERIAL EYE	4	3	-	-
CONJUNCTIVITIS	2	-	-	-
GASTROINTESTINAL	2	2	1	3
PERIANAL STAIN/FECES	2	1	1	1
STAIN MOUTH	-	-	-	2
DIARRHEA	-	1	-	1
GENERAL	-	-	-	1
DEHYDRATED	-	-	-	1

* Data presented as number of animals exhibiting the sign.

TABLE 7: Serum Chemistry Summary*

Group	Baseline	Control	Control	2
Day	0	180	210	180
n	9	10	5	10
ACHE	1.433	1.484	1.534	1.175@
U/ml	±0.197	0.206	0.305	0.204
CHE	0.337	0.437	0.318	0.326@
U/ml	±0.081	0.101	0.051	0.129
ALT	56.58	64.91	46.00	69.42
U/l	±9.61	19.25	6.48	20.15
AST	138.67	143.65	96.12	122.64
U/l	±64.79	22.28	19.16	27.51
ALK	254.66	124.31	82.60	131.38
U/l	±55.92	49.07	37.69	50.26
LDH	601.80	1232.71	674.44	910.63
U/l	±305.44	496.83	577.83	341.50
CK	853.17	794.50	566.68	547.02
U/l	±386.24	371.12	228.71	99.55
BILI	0.00	0.00	0.00	0.00
mg/dl	±0.00	0.00	0.00	0.00
CHOL	58.26	81.24	72.32	76.69
mg/dl	±10.97	21.92	6.57	19.35
TRIG	91.99	251.94	234.60	264.03
mg/dl	±33.65	102.32	92.34	73.78
URIC	2.44	3.01	1.98	2.57
mg/dl	±0.50	1.44	0.87	0.52
TP	5.14	6.50	6.06	6.49
g/dl	±0.25	0.22	0.30	0.44

* Data are presented as the mean ± the standard deviation.
@ Significant difference from controls at $p \leq 0.05$.

TABLE 7 (cont.): Serum Chemistry Summary*

Group Day n	3 180 10	7 180 10	3A 210 5	7A 210 5
ACHE U/ml	0.756@ ±0.214	1.021@ 0.271	1.728 0.215	0.984@ 0.181
CHE U/ml	0.160@ ±0.040	0.304@ 0.079	0.334 0.053	0.340 0.080
ALT U/l	78.51 ±29.79	91.27 65.52	53.58 8.45	54.16 10.64
AST U/l	147.21 ±46.87	168.90 80.31	126.58 16.24	142.30@ 26.42
ALK U/l	106.76 ±24.26	106.06 29.82	98.56 8.93	102.10 38.45
LDH U/l	1006.58 ±326.72	986.19 518.27	1478.58@ 582.30	1482.40@ 165.08
CK U/l	607.95 ±265.18	680.11 283.40	924.50@ 135.33	1012.50@ 219.02
BILI mg/dl	0.00 ±0.00	0.00 0.00	0.00 0.00	0.00 0.00
CHOL mg/dl	78.41 ±14.59	79.08 13.15	71.02 22.51	78.40 12.59
TRIG mg/dl	280.61 ±72.05	251.99 128.23	206.32 60.51	275.62 103.52
URIC mg/dl	2.37 ±0.96	3.09 1.99	2.94 1.17	1.82 0.40
TP g/dl	6.37 ±0.29	6.35 0.36	6.18 0.26	6.06 0.17

* Data are presented as the mean ± the standard deviation.
@ Significant difference from controls at $p \leq 0.05$.

TABLE 7 (cont.): Serum Chemistry Summary*

Group Day n	Baseline 0 9	Control 180 10	Control 210 5	2 180 10
ALB g/dl	2.776 ±0.208	3.223 0.224	3.348 0.133	3.207 0.224
GLU mg/dl	237.83 ±29.79	266.60 54.51	236.90 24.44	256.69 42.42
BUN mg/dl	15.84 ±2.53	18.89 2.71	16.18 1.15	16.78 1.89
CR mg/dl	0.451 ±0.079	0.690 0.099	0.608 0.120	0.625 0.048
CAL mg/dl	10.98 ±0.56	10.25 0.30	10.12 0.40	10.12 0.32
PHOS mg/dl	9.833 ±0.870	6.197 0.817	5.542 0.395	6.278 0.702
NA Meq/l	146.3 ±3.0	146.3 2.0	147.8 0.4	144.3 1.9
CL Meq/l	101.8 ±2.2	102.1 1.7	101.0 1.4	103.3 3.3
K Meq/l	6.50 ±0.43	6.81 1.23	6.42 0.70	6.55 0.84
IRON µg/dl	287.3 ±106.6	146.7 22.9	123.4 23.8	155.1† 13.7
MAG mg/dl	2.723 ±0.131	2.636 0.432	2.342 0.248	2.529 0.190

* Data are presented as the mean ± the standard deviation.
† Number of animals per group, n, = 9.

TABLE 7 (cont.): Serum Chemistry Summary*

Group	3	7	3A	7A
Day	180	180	210	210
n	10	10	5	5
ALB g/dl	3.200 ±0.224	3.252 0.209	3.354 0.147	3.340 0.126
GLU mg/dl	242.19 ±31.14	257.12 71.04	232.52 16.82	231.18 20.09
BUN mg/dl	19.53 ±2.92	18.25 1.66	17.94 2.30	16.50 1.30
CR mg/dl	0.652 ±0.106	0.688 0.042	0.604 0.063	0.548 0.216
CAL mg/dl	9.94 ±0.39	10.82@ 0.44	10.24 0.64	10.06 0.23
PHOS mg/dl	6.188 ±1.084	6.975 0.738	5.526 0.317	5.438 0.609
NA Meq/l	144.9 ±0.9	148.3 2.2	148.2 1.1	148.4 1.5
CL Meq/l	104.2 ±1.3	103.0 2.1	100.0 0.7	98.8@ 0.4
K Meq/l	6.03 ±0.58	6.73 1.19	7.40 0.91	7.00 0.85
IRON µg/dl	162.2 ±27.7	148.7 13.3	151.2 29.3	140.2 23.6
MAG mg/dl	2.312 ±0.277	2.752 0.482	2.684 0.166	2.642 0.231

* Data are presented as the mean ± the standard deviation.
@ Significant difference from controls at $p \leq 0.05$.

TABLE 8: Hematology Summary*

Group Day n	Baseline 0 10	Control 180 10	Control 210 5	2 180 8
RBC x10^6/µl	5.925 ±0.672	7.942 0.447	7.998 0.310	7.665 1.278
HGB g/dl	13.15 ±1.61	14.78 0.37	14.80 0.35	14.08 2.33
HCT %	37.04 ±4.26	40.82 1.61	40.04 1.15	39.24 7.20
MCV fl	61.0 ±1.3	50.6 1.0	50.0 1.4	50.6 1.7
MCH pg	22.27 ±0.74	18.40 0.42	18.56 0.46	18.44 0.66
MCHC g/dl	35.43 ±0.66	36.24 0.82	36.98 0.19	36.14 1.60
PLT x10^3/µl	795.7 ±144.6	NT	911.2 70.4	NT
WBC x10^3/µl	5.10 ±1.39	7.17 1.84	7.42 1.59	7.23 2.20
SEG %	17.0 ±8.4	15.4 8.4	21.2 14.3	22.0 9.4
EOS %	0.2 ±0.4	0.3 0.7	0.6 0.5	0.4 0.7
BAS %	0.0 ±0.0	0.0 0.0	0.0 0.0	0.0 0.0
LYM %	82.5 ±8.0	84.1 9.2	78.2 14.0	76.6 9.5
MON %	0.3 ±0.5	0.2 0.6	0.0 0.0	0.0 0.0

* Data are presented as the mean ± the standard deviation.

TABLE 8 (cont.): Hematology Summary*

Group Day n	3 180 9	7 180 9	3A 210 5	7A 210 5
RBC x10^6/µl	7.626 ±0.990	8.061 0.429	8.390 0.159	7.594 0.660
HGB g/dl	14.62 ±0.62	14.68 0.84	15.40 0.25	14.76 1.18
HCT %	40.01 ±1.58	40.74 2.25	41.68 0.52	39.80 3.40
MCV fl	49.4 ±1.2	50.6 1.6	49.6 1.1	50.8 2.9
MCH pg	18.17 ±0.55	18.24 0.57	18.44 0.46	18.96 1.00
MCHC g/dl	36.61 ±0.56	36.07 0.60	37.00 0.26	37.14 0.43
PLT x10^3/µl	NT	NT	892.8 105.6	758.8 443.9
WBC x10^3/µl	7.40 ±1.75	6.87 2.53	6.24 1.07	7.24 3.27
SEG %	16.1 ±11.2	13.7 5.3	14.2 7.7	20.8 10.2
EOS %	0.7 ±1.0	0.3 0.7	0.8 0.8	0.0 0.0
BAS %	0.1 ±0.3	0.0 0.0	0.0 0.0	0.0 0.0
LYM %	83.0 ±11.2	85.8 5.2	82.8 7.2	78.8 9.8
MON %	0.1 ±0.3	0.2 0.4	0.2 0.4	0.4 0.5

* Data are presented as the mean ± the standard deviation.

TABLE 9: Percent Cholinesterase Inhibition*

Group		Study Day 180	210
1	ACHE	0.0	0.0
	CHE	0.0	0.0
2	ACHE	20.8	
	CHE	25.4	
3/3A	ACHE	49.1	-12.6
	CHE	63.4	-5.0
7/7A	ACHE	31.2	35.9
	CHE	30.4	-6.9

* Percent inhibition calculated as [(mean baseline activity - normalized mean treated activity) ÷ mean baseline activity] x 100%.
Normalized mean treated activity calculated as (mean baseline activity ÷ mean control group activity) x mean treatment group activity.

@ Negative percent inhibition indicates treatment group activity level exceeded the baseline activity.

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Appendices

Appendix A: CHEMICAL DATA

Chemical Name: Pyridostigmine bromide

Other Names: 3-[[(Dimethylamino)carbamyl]oxy]-1-methylpyridinium bromide, 3-hydroxy-1-methylpyridinium bromide dimethylcarbamate, 1-methyl-3-hydroxypyridinium bromide dimethylcarbamate, 3-(dimethylcarbamyloxy)-1-methylpyridinium bromide

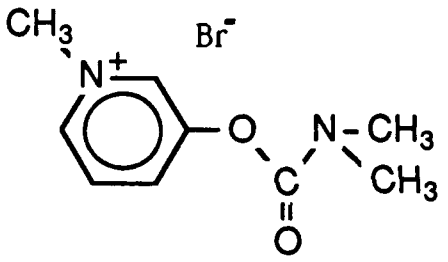
Chemical Abstracts Registry Number: 101-26-8

Lot Number: 590034

LAIR Code Number: TW71A

WRAIR Code Number: WR-250710AF

Chemical Structure:



Molecular Formula: $C_9H_{13}BrN_2O_2$

Molecular Weight: 261.13

Analytical Data:

The test compound was analyzed by the sponsors using HPLC, elemental analysis, and UV spectroscopy.[1] This data verified the identity of the compound and provided the following estimates of purity: 99.6% (by HPLC), 98% (by elemental bromide), and 100% (by UV spectroscopy).

Pyridostigmine bromide was analyzed in this lab by NMR[2] and HPLC[3].

Appendix A (cont.): CHEMICAL DATA

NMR (300 MHz, D_2O): d 3.02, 3.16 (singlets, $(CH_3)_2$-N-, 6 H); 4.43 (singlet, CH_3-N(pyr), 3H); 8.09 (quartet, J = 8.6, 6.3 Hz, O-C=CH-C*H*=CH-N, 1 H); 8.39 (doublet, J = 9.0 Hz, O-C=C*H*-CH-, 1 H); 8.71 (doublet, J = 6.0 Hz, CH=C*H*-N, 1 H); 8.86 (singlet, O-C=C*H*-N, 1 H). No other signals were observed in the spectrum.

HPLC analysis of the compound was performed using a Hewlett-Packard 1090 HPLC equipped with a diode array detector. The compound was chromatographed under the following conditions: column, silica (Brownlee Labs, Inc., 4.6 x 100 mm); mobile phase, 80% buffer (0.01 M heptane sulfonic acid, 0.01 M sodium dihydrogen phosphate, 0.0025 M tetramethylammonium chloride, pH adjusted to 3 with sulfuric acid)/20% acetonitrile; flow, 1.5 ml/min; wavelength monitored, 269 nm. Under these conditions, pyridostigmine bromide eluted as one peak at 2.4 min. No other peaks were present in the chromatogram.

The data obtained in our lab confirm the identity and high purity of the test compound.

Source: Mr. William Ellis
Division of Experimental Therapeutics
Walter Reed Army Institute of Research
Washington, DC
Requested by LTC William Ritter, WRAIR

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[1] Petesch R, Benitez A and Lim P. Assay of pyridostigmine bromide, WR-250710AF, BK75309, lot no. 590034. Menlo Park, California: SRI International, 3 July 1984; Draft report no. 476.

[2] Wheeler CR. Toxicity testing of antidotes of chemical warfare agents. Laboratory notebook #85-12-024.1, p. 70-71. Letterman Army Institute of Research, Presidio of San Francisco, CA.

[3] *Ibid.* p. 72-74.

Appendix B: ANIMAL DATA

Species: *Rattus norvegicus*

Strain: Sprague-Dawley

Source: Charles River Laboratories, Inc.
Charles River Portage
Shaver Road
Portage, Michigan 49081

Sex: Male

Date of birth: 15 September 1986

Method of randomization: Random number generating program (LAIR SOP OP-ISG-21)

Animals in each group:

Group 1	15
Group 2	10
Group 3/3A	10/5
Group 7/7A	10/5

Condition of animals at start of study: Normal

Body weight range at start of dosing: 141 - 272 g

Identification procedures: Tail tattoo (SOP OP-ARG-1)

Pretest conditioning: Quarantine/acclimation from 21 October - 3 November 1986

Justification: The laboratory rat has proven to be a sensitive and reliable system for sub-chronic oral toxicity determination.

Appendix C: SUBCHRONIC TOXICITY TESTING IN RODENTS

OP-STX-74
PAGE 1 of 9
15 May 1987
Replaces 21 Jun 85

TITLE: Subchronic Toxicity Testing in Rodents

SCOPE: This subchronic toxicity study is designed to assess the toxic potential of a test substance when administered to a rodent for between 28 and 180 consecutive days. It is conducted in compliance with the Toxic Substance Control Act as administered by the EPA. This study will also be conducted in compliance with the Good Laboratory Practices regulation promulgated by the FDA.

REFERENCES:

1. EPA, Toxic Substances Control, GLP Standards; Final Rule, (40 CFR 792) 29 Nov 83, (48 FR 53922-53944).

2. EPA, Pesticide Programs, GLP Standards; Final Rule, (48 CFR 160) 29 Nov 83 (48 FR 53946-53969).

3. FDA, Nonclinical Laboratory Studies (21 CFR 58) Final Rule, 22 Dec 78 (43 FR 59986-60025), as amended 11 Apr 80 (45 FR 24865).

4. EPA, Health effects test guidelines. Office of Pesticides and Toxic Substances. EPA 560/6-82-001.

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PROCEDURE:

A. Study Design

1. Species: A recognized strain of the laboratory rodent (e.g., rat; Sprague-Dawley, Fisher-344) will be purchased from a licensed dealer by the Division of Animal Care and Service, LAIR. Generally the strain selected should be one which will be utilized in chronic studies.

Appendix C (cont.): SUBCHRONIC TOXICITY TESTING IN RODENTS

2. Age and Sex: Equal number of males and females will be tested. Animals should receive the initial dose before attaining eight weeks of age.

3. Number of Animals: Each group, treatment and concurrent control, must contain a minimum of 10 animals/sex/group. If interim sacrifices are required the number of animals will be increased by the number scheduled for sacrifice.

4. Quarantine: Animals will be quarantined for two weeks in the GLP Suite for environmental acclimatization and detection of disease/parasites/poor health. Randomly selected animals (2% of total) will be sacrificed by the pathologists for quality control.

5. Animal Identification and Randomization: Animals will be identified by tail tattoo and randomly assigned to control and treatment groups. Randomization will be by a weight stratification procedure.

6. Husbandry: Animals will be housed individually in shoe-box cages in the GLP Suite. Temperature in the GLP Suite will be maintained in the range of 72 - 76' F with relative humidity of 40-60%. A 12 hour light/dark cycle will be utilized. Temperature and relative humidity will be recorded. Animals will be fed batch certified rodent chow ad libitum and provided deionized reverse osmosis treated water ad libitum. The deionized reverse osmosis water is periodically analyzed on a retrospective basis. None of the contaminants in food and water are reasonably expected to be of sufficient titre as to interfere with conduct of the proposed studies.

7. Control Group(s): A vehicle control group will be used, and in cases where the effects of the vehicle are unknown, an untreated control group will also be tested.

8. Treatment Groups: At least three dose levels will be tested. The highest dose level should produce toxicological or pharmacological effect but produce no more than 10 percent lethality. This dose should be higher than that expected for human exposure. The low dose should not produce evidence of toxicity. The intermediate dose should be a multiple of the low dose and provide an estimate of the dose-response relationship. Generally, a limit dose of 1000 mg/kg will be the maximum dose level used, unless higher dose levels are justified.

Appendix C (cont.): SUBCHRONIC TOXICITY TESTING IN RODENTS

9. Duration: The test substance will be administered for between 28 and 180 consecutive days.

10. Administration of Test Substance: The test substance will be administered in the diet at a constant concentration (ppm) unless oral gavage or diet administration (constant dose, mg/kg) is required by the Sponsor. The constant dose procedures requires weekly adjustment of dose because of changing body weights. Other routes, such as subcutaneous,dermal patch, I.V., etc., may be used if they are more representative of the potential route of exposure or administration.

11. Test Substance Analysis and Stability: Physio-chemical data on the batch or lot of the test substance used in the study will be provided by the sponsor as well as an analytical profile of major constituents and/or contaminants/impurities. Safety precautions will also be provided by the sponsor. Stability of the test substance in the diet (vehicle) and homogeneity for the range of concentrations used will be determined at the initiation of the study. Additionally, assays for homogenicity will be run at selected intervals during conduct of the study. Assays for concentration will be conducted on each dosing mixture prepared.

12. Quality Assurance: The LAIR Quality Assurance Unit will audit the protocol, in-life phase, and final report for compliance with GLP procedures.

B. Study Conduct

1. Observations: All toxicological and pharmacological signs will be recorded daily, including time of onset, intensity and duration. Food and water consumption will be measured and animals will be weighed weekly.

2. Clinical Laboratory Testing: At least 5 animals/sex/group will be bled at selected intervals during the study and at termination of the study. The same animals should be bled on each occasion if possible. At the discretion of the sponsor and/or study director, clinical laboratory testing may be done by serial sacrifice.

a. Hematologic evaluation - The PCV, Hgb, RBC, WBC, differential, MCV, MCHC, and platelets determinations are required. If signs of anemia are present, reticulocyte counts will be performed on whole anticoagulated blood.

Appendix C (cont.): SUBCHRONIC TOXICITY TESTING IN RODENTS

b. Blood Chemistry - Ca, Na, K, Mg, Cu, total Fe, LDH, serum ALT, serum AST, glucose, BUN, direct/total bilirubin, A/G ratio, cholesterol, albumin, globulin, total protein, Cl, uric acid, creatinine, CPK, methemoglobin, P, and triglycerides will be measured.

c. Cholinesterase inhibition - If the sponsor indicates that the test substance may inhibit acetylcholinesterase activity, plasma and erythrocyte acetylcholinesterase activity will be monitored at selected intervals during the study.

d. Urinalysis - If applicable the following will be measured before the initiation of dosing, during the seventh week, and near the termination of the study:

1. specific gravity (osmolarity)
2. pH
3. protein
4. ketones
5. glucose
6. bilirubin
7. urobilinogen
8. occult blood
9. microscopic observation of casts, etc.

3. Moribund animals should be sacrificed and a complete necropsy and tissue/blood collection performed, to lessen the likelihood of unobserved death and post mortem autolysis. Animals found dead will be subjected to a gross necropsy. Histopathology on these animals will be at the discretion of the Pathologist.

4. Gross Necropsy

a. All animals are subjected to gross necropsy and examination of external surface, all orifices, cranial cavity, external and cut surfaces of the brain and spinal cord, the thoracic, abdominal and pelvic cavities and their viscera; the cervical tissues and organs, and carcass.

Appendix C (cont.): SUBCHRONIC TOXICITY TESTING IN RODENTS

b. The following tissues are weighed:

liver

kidneys

inrenals

heart

gonads

brain

5. Histopathology

a. Animals in vehicle and cage control and high dose groups will have histopathology performed on:

brain (3 levels)

eye

pituitary

salivary gland

heart

thymus

thyroid/parathyroid

lung w/mainstem bronchi

trachea

esophagus

stomach

small and large intestine

adrenals

Appendix C (cont.): SUBCHRONIC TOXICITY TESTING IN RODENTS

pancreas

liver

kidneys

urinary bladder

testes

prostate

ovaries

uterine horn and corpus

spleen

bone (with marrow) from sternebrae, vertebrae, tibio-femoral joint

skeletal muscle

all gross lesions

b. Low and intermediate dose groups will have histopathology performed on liver, lung, kidney, heart, any gross lesion and any target organ (determined from either the high dose or from laboratory tests).

6. Data Reporting and Evaluation

a. Animal records will be arranged by dose level and sex. All means accompanied by standard deviation and/or standard error of the mean will be reported.

b. In tabular form data must be provided, as follows, for each animal.

1. Identification number

2. Status at and date of death

Appendix C (cont.): SUBCHRONIC TOXICITY TESTING IN RODENTS

3. Age at beginning of study

c. Toxic, pharmacologic and behavioral effects for each animal and each group.

1. A list of each sign of toxicity affecting any animal

2. Number of animals affected

3. The median time for development of such responses

4. Weekly survival and sacrifice data

d. Food consumption and body weight data: for each animal, the following should be tabulated:

1. Identification number

2. Weekly measured food consumption

3. Weekly body weight

4. Food and body weight means for each group

5. If compound mixed with diet, weekly compound consumption per group.

e. Clinical laboratory tests results:

1. Rationale for timing if different from this SOP.

2. Rationale and method for selection of animals for clinical laboratory tests.

3. Results by animal and by group.

f. Gross anatomy results by test group in tabular form

1. Data on gross abnormalities, description by animal and group.

2. For each individual, body weight, organ weight, and organ to body weight ratio, mean weights of each type of organ, mean organ to body weight ratio.

Appendix C (cont.): SUBCHRONIC TOXICITY TESTING IN RODENTS

g. Histopathology data arranged by test group:

1. For each animal, its identification number and complete description and diagnosis of every lesion in the animal. Abnormalities observed repeatedly need to be described only once and may subsequently be supplied by reference, with any individual variation noted as necessary.

2. For each animal a table or paragraph listing tissues found to be normal.

3. If a grading system is used, a description of the system.

4. Counts and incidence of lesions by test groups. In tabular form for each test group:

a. The number of animals at the start, and number of animals in which any lesion was found.

b. The number affected by each different type of lesion, the number examined for each type, the percentage of animals examined that were affected.

c. The number of different types of lesions.

5. Observance of tumors will necessitate the inclusion of a complete description and diagnosis of each tumor.

h. Data Evaluation: An evaluation of the test results, including the statistical analyses, based on clinical findings, gross necropsy findings and histopathology results will be made. It will include the evaluation of the relationship of the animal's exposure to the test substance and the incidence and severity of all abnormalities, gross and histological changes, organ weight changes, effects on mortality and other toxic effects. It should include dose response curves for effects that appear compound related and description of statistical methods.

Appendix C (cont.): SUBCHRONIC TOXICITY TESTING IN RODENTS

OP-STX-74
PAGE 9 of 9
15 May 1987

Approved: 7 APR 87 (Date)

DON W. KORTE JR., PHD
MAJ, MS
Chief, Toxicology Branch

Certified: 13 May 87 (Date)

CAROLYN M. LEWIS
DAC
Quality Assurance Officer

Appendix D: HISTORICAL LISTING OF STUDY EVENTS

Date	Event
21 Oct 86	Study animals arrived at LAIR. They were sexed, observed for illness, weighed, and caged in the GLP Suite.
22 Oct 86	Four study animals were submitted for quality control necropsy.
22,24,29 Oct 86	Study animals were tattooed.
22 Oct - 3 Nov 86	Study animals were checked daily.
28 Oct 86	Study animals were weighed and food and water consumption monitored (feeders and water bottles weighed).
29 Oct 86, 1 May,1 Jun 87	Ophthalmic examinations were performed at baseline and within 1 week prior to sacrifice.
4 Nov 86	Animals were removed from quarantine and weighed, dietary concentrations were calculated, and diet containing test compound was started. Ten baseline control animals were submitted for necropsy, hematology, serology, and cholinesterase determinations.
4 Nov 86 - 1 Jun 87	Observations were conducted twice daily.
11,18,25 Nov, 2,9,16,23,30 Dec 86,6,13, 20,27 Jan,3, 10,17,24 Feb, 3,10,17,24,31 Mar,7,14,21,28 Apr,5,12,19,26 May 87	Males were observed and weighed, and water bottles and feeders were weighed. Diet requirements were recalculated and new feed mixes prepared. Feeders were changed to new mix.
5,7 May 87	Ten males from Groups 1, 2, 3, and 7 were submitted for necropsy, hematology, serology, and cholinesterase determinations.
2 Jun 87	Five males from Groups 1, 3A, and 7A were submitted for necropsy, hematology, serology, and cholinesterase determinations.

Appendix E: PROCEDURES FOR DIET PREPARATION

OP-STX-16
Page 1 of 16
20 May 1988
REPLACES: 1 November 1986

TITLE: Diet Preparation for Feeding Studies

SCOPE: These procedures comply with the FDA and EPA GLP Regulations and are applicable to preparation of diets for studies in which the test chemical is administered in the food.

REFERENCES:

1. EPA, Toxic Substances Control, GLP Standards (40 CFR 792). Final Rule, 29 Nov 83, (48 FR 53922-53944).

2. EPA, Pesticide Programs, GLP Standards (40 CFR 160) Final Rule, 29 Nov 83 (48 FR 53946-53969).

3. FDA, Nonclinical Laboratory Studies (21 CFR 58) Final Rule, 22 Dec 78 (43 FR 59986-60025).

4. EPA, Health effects test guidelines. Office of Pesticides and Toxic Substances. EPA 560/6-82-001.

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Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

INTRODUCTION:

Overview

Diet preparation consists of four stages: initial testing, target concentration calculations, premix preparation and diet preparation. The time required for each of these stages depends in part on the methods used and on the number of diets prepared. From start to finish, diet preparation for one feeding period can take from one to four days.

Safety Precautions

Certain precautions should be taken to prevent worker exposure and contamination of other areas outside the diet mixing room when preparing the premix and the diet. People preparing diet should be familiar with LAIR SOP OP-STX-69, "Safety Procedures for Handling of Test Compound and Positive Control Carcinogens". The risk of exposure when making premix in the jar mill may not be as great as when making diet in the open mixers since it is not as likely to become airborne, but one should still be cautious when handling the premix since the concentration of the test chemical is much greater. With highly toxic chemicals or suspect carcinogens, individuals preparing the premix should at least wear gloves, disposable gown, and shoe coverings. Individuals preparing the diet should also wear a mask and head covering. The type of gloves, gown, and mask worn will depend on the physical nature of the chemical and the degree of toxicity or carcinogenicity of the chemical. With chemicals of low toxicity these measures are optional. The safety officer for the group should be consulted if there are any questions regarding the appropriate clothing to wear. The protective clothing should not be worn outside the diet mixing area to prevent contamination of other areas. To minimize contamination, it is recommended that the door to the diet mixing room be closed and locked during diet preparation. The jar mill and open mixers should be used in the hood with the blower on.

Cleaning up is also critical for keeping contamination to a minimum. After completing the diet preparation, the equipment should be cleaned including the jar mills, grinding pellets, mixing bowls, beaters, V-type blender, weighing containers, scoops and spatulas. In addition, the counter tops should be cleaned and the floor swept or vacuumed. If a vacuum cleaner is used it should have a HEPA filter on the exhaust. The floor should be cleaned with a wet mop as needed.

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

PROCEDURES:

I. Initial Testing

Before a feeding study starts the following needs to be done: validate the adequacy of the cleaning procedures, determine the stability of the test compound in the feed, and verify the homogeneity of the test compound/feed mixtures.

A. Validating the Cleaning Procedures

1. After mixing the test compound with the feed at the highest concentration to be used during a study, wash the blender or mixer. The recommended procedure for washing the blenders/mixers is to fill them about a third full with water and add approximately 10-30 mls of laboratory glassware detergent. Blenders, like the Patterson-Kelley blenders, which have mixing containers that cannot be easily removed for cleaning should be turned on for no more than 1 minute after adding the soapy water. Be sure the blender is sealed tightly so that the water does not leak out. The mixers or blenders should then be rinsed at least twice with water (the volume equal to the wash volume). The blenders will be turned on for approximately 1 minute per rinse.

2. These procedures can be modified. The procedures used to clean the mixers and blenders should be documented in the cleaning log book each time they are cleaned, regardless of the method used. The type of detergent, solvent, and number of rinses should be recorded.

3. After cleaning the mixer or blender, it will be checked for residual test compound. A small amount (10-50 mls) of an appropriate solvent (water, methanol, ethanol, isopropanpol, etc.) will be added. The blender or mixer will be turned on for no more than 1 minute. The solvent wash will be analyzed by appropriate method (HPLC, GC, etc.). The solvent and method of analysis used will be documented.

B. Stability Determination

The stability of the test compound in the feed should be determined for a period of time no less than the time from which the diet is prepared to the time it is removed from the feeders. The stability should be tested at concentrations which bracket the range of concentrations

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

that will be used in the study. For additional information refer to OP-STX-95, "Analytical Chemistry Requirements for Toxicity Testing of Chemicals."

C. Homogeneity Verification

The adequacy of the mixing procedures is verified by preparing the test compound/feed mixtures at concentrations which bracket the concentrations to be used in the study. The volume of the mixtures prepared should also bracket the volumes to be used in the study. Homogeneity is tested by removing samples from the top, middle, and bottom of the mixing bowl or from each of the three ports of the Patterson-Kelly blenders. Samples will be analyzed by the appropriate method for the test compound (HPLC, GC, etc.). The concentrations of each sample should deviate no more than 10% from the mean of the three samples. For additional information refer to OP-STX-95, "Analytical Chemistry Requirements for Toxicity Testing of Chemicals." Homogeneity should be re-checked periodically during the course of the study.

II. Calculation of Target Concentration for Diets

The time required for calculating the target concentration for each dose group can vary depending on whether they are done automatically by the TOXSYS programs or manually. If the TOXSYS programs are used, the calculations can be done on the same day the diets are blended. If the calculations are performed manually, they should to be done at least one day before the diets are blended.

A. Place the animals on the powdered feed (control diet) during quarantine. Record feeder weights on TOXSYS IAW SOP OP-ISG-17, "Standard Procedures for Acquiring Toxicology Experiment Data Using a TOXSYS Data Collection Terminal" or manually (Figure 1). If recorded manually, calculate the net food consumed for each animal by subtracting the old feeder weight from the previous new feeder weight. Record the net food consumed on the form in Figure 1.

B. Determine the mean daily food consumption for each group during the baseline period. If TOXSYS is used, the calculations described in steps B - F are done automatically with the DIET Program (SOP OP-ISG-36, Standard Procedures for Computing Diet Mix Concentration on a TOXSYS Data Collection Terminal) on the TOXCART or with the DIETPREP program (SOP OP-ISG-33, Standard Procedures for Reporting Animal Data Base Records on the LAIR Central Computer) on the mainframe computer. If the calculations are done manually,

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

record your results from steps B - F on the form in Figure 2. To calculate the mean daily food consumption manually, average the net food consumed by all the animals in a group and divide that by the number of days in the dose period.

C. Weigh the animals at the end of the same week that baseline food consumption data are collected during quarantine. Use the data to calculate the mean body weights for each group.

D. Multiply the dose level (mg/kg/day) for each group by the mean body weight (kg) and divide by the corresponding mean daily food consumption (g/day) to obtain the target concentration (mg/g) of the diet for that group.

E. Multiply the target concentration by the correction factor to allow for changes in the ratio of the mean body weight and the mean food consumption that occur as a result of growth. The correction factor is based on historical data from animals of the same species and strain which are of similar age and have been fed a similar diet. If no data is available set the correction factor equal to 1 which will not affect the target concentration.

F. At the end of each week or feeding period recalculate the mean daily food consumption and the mean body weight for each group based on the feeder weights and body weights recorded during that period.

G. Recalculate the target concentration for each group using the new mean daily food consumption and new mean body weight.

H. If an animal dies during a feeding period, do not use it in the calculations. In addition, do not include animals whose food consumption is questionable due to some unusual circumstance, such as significant spillage of food or wet food.

III. Premix Preparation

The premix may be made several ways. If the test chemical is a solid that is stable in the feed, then a premix can be made up several days in advance and can be used with all the diets. If the dosing range is too large (greater than a 100 fold difference between low and high dose levels), then 2 or more premixes may be needed. In this situation, making separate premixes for each diet may be easier. Procedures for both methods are given below.

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

A. Preparation of a Single Premix for All Diets

If a single premix is prepared for all diets, then the premix should be prepared at least one day prior to diet preparation. The time required for premix preparation will depend on whether the chemical needs to be ground in the jar mill. If the jar mill is used, premix preparation may require 4 - 8 hours.

1. Calculation of Premix Concentration

   a. For the first dose period approximate the diet concentration for the each dose group by using estimates of the mean daily food consumption and the mean body weight. For subsequent dose periods use the diet concentrations from the previous period. Select a concentration for the premix that is at least 2 - 3 times greater than the diet concentration of the high dose group.

   b. Calculate the amount of diet needed for each group by multiplying the number of animals per group times the mean daily food consumption times the number of days per period times 1.5 to allow for wastage.

   c. Approximate the amount of test chemical needed by multiplying the amount of diet needed for each group (g) by its concentration.

   d. To determine the amount of premix needed, divide the amount of test chemical needed (g) by the concentration of the premix (mg/g). Add at least another one third more to this amount to allow for increases in the diet concentrations due to animal growth.

2. Blending of Premix

   a. Calibrate the balances(s) to be used for preparing the premix and record the weights in the appropriate log book(s). Record the LAIR ID number (4-digit number) of the balance on the form in Figure 3.

   b. Weigh out the desired amount of test chemical on the balance. Record the weight and lot number of the test chemical on the form in Figure 3.

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

c. Transfer the test chemical to the bowl of a mixer. NOTE: If the test chemical is coarsely ground or in clumps, transfer it to the porcelain jar of the jar mill. Add the porcelain grinding pellets. Grind the test chemical alone for at least 15 minutes.

d. Weigh out the amount of feed needed to achieve the desired concentration. Record the weight and the lot number of the feed. Record the LAIR ID number for the balance, if different from the one above.

e. Add a portion of the feed roughly equal to the weight of the test chemical to the mixer or jar mill. Stir with a spatula. If the premix is prepared in the jar mill, grind at least 15 minutes. Repeat this step, doubling the amount of feed added, until all the feed has been added.

f. Mix the premix with the mixer for 15 minutes in the hood. If the jar mill is used, grind another 15 minutes after the last addition of feed.

g. Remove at least a 10 g sample from the premix. Part of the sample is for analysis and the remainder is for archival.

h. Transfer the rest of the premix to a plastic bag and label it clearly with the study number, date, chemical, concentration and your initials. If the premix was prepared in the jar mill, be sure to remove all of the grinding pellets from the premix since they can be harmful to the blender if not detected before adding the premix to the feed (not to mention its effect on the concentration). The easiest way to remove them is to sift the premix through a large mesh sieve when transfering it to the bag.

B. Preparation of Separate Premixes for Each Diet

This method is recommended when using a liquid, hygroscopic, or unstable test chemical or when the range of dose levels is large.

1. Blending of Premixes with Solid Chemicals

a. Calibrate balances(s) to be used in premix

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

preparation and record weights in log book(s). Record the LAIR ID number(s) (4-digit number) of the balance(s) on the form in Figure 4.

b. Record lot numbers of the test chemical and feed on the form in Figure 4.

c. Accurately weigh out the test chemical and transfer it to a large beaker (600-2000 ml) or a large mortar if the chemical is coarsely ground or in clumps. Record the weight on the form.

d. Weigh out the total amount of feed to be added to the premix and record the weight on the form. The total weight of the premix should be at least 10% of the total weight of the diet.

e. From the feed that has been weighed out, take an amount that is roughly equal to the weight of the chemical and add it to the chemical, mixing or grinding afterwards.

f. Add more feed and mix or grind in, doubling the amount of feed added each time until all the feed has been added.

g. Transfer to the bowl for the small mixer and mix at low speed for at least 5 minutes in the hood.

2. Blending of Premixes with Liquid or Hygroscopic Chemicals

a. Calibrate balances(s) to be used in premix preparation and record weights in log book(s). Record the LAIR ID number(s) (4-digit number) of the balance(s) on the form in Figure 4.

b. Record lot numbers of the test chemical and feed on the form in Figure 4.

c. Accurately weigh out test chemical into a small (50-200 ml) beaker. Record weight on the form. NOTE: If the chemical has large clumps or is coarsely ground, weigh out the chemical on a weigh boat and transfer to a mortar. Grind the chemical alone before adding any feed.

d. Weigh out the total amount of feed to be

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

added to the premix and record the weight on the form. The total weight of the premix should be at least 10% of the total weight of the diet.

e. Transfer roughly 100 g of the feed that has been weighed to a USS No. 100 mesh sieve and shake until approximately 10g of feed flour have been collected.

f. Add approximately 1 g of feed flour to the beaker or mortar containing the test chemical and mix or grind. Continue to add 1-2 g increments of flour until all the feed flour has been added. If a weigh boat was used, add the increments of feed to the weigh boat first. Stir with spatula and transfer to the mortar.

g. Transfer the mixture to the bowl for the small mixer. Add the coarse feed left on the sieve in increments of 25-50 g, stirring the coarse feed in the beaker or mortar first before adding to the bowl. Stir with a spatula after each addition.

h. Add the remaining feed in increments of roughly 100 g, stirring with a spatula after each addition.

i. Mix for at least 5 minutes on low speed in the hood.

IV. Blending of Diets

The type of blender used will depend on the toxicity/carcinogenicity of the test chemical. If the chemical is highly toxic or suspected of being carcinogenic, the diet should be prepared in the Patterson-Kelley V-type blender which is closed. Should it be necessary to use an open blender like the Hobart with a highly toxic/carcinogenic test chemical, use the blender in the hood. If an open blender is used with a low or moderately toxic chemical, place a large (preferably clear) plastic garbage bag over the blender when mixing to minimize the amount of diet that becomes airborne during mixing.

Each diet requires at least 45 minutes to an hour to prepare, allowing for set-up and clean-up time. Depending on how many diets there are to prepare, it may require more than one day to complete this stage.

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

A. Calibrate the balance(s) to be used for diet preparation and record weights in the appropriate log book.

B. Calculate the amount of feed that should be added to the premix to get the desired concentration and divide in half. Weigh out the feed into two separate containers (i.e., one half of total into each). Record the weights on the form in Figure 5. Record the lot number and the LAIR ID number(s) (4-digit number) for the balance(s) on the form, too.

C. If using a single premix prepared ahead of time, weigh out the desired amount of premix and record it on the form. Record the date of the premix and the balance used (if different from the one above).

D. The procedure for mixing the diet will depend on the blender selected.

1. Hobart or Open Type of Blenders

   a. Transfer half of the feed to the mixing bowl. Add the premix on top of the feed in the mixing bowl, then add the other half of the feed on top of the premix.

   b. Mix the diet in the mixer for at least 15 minutes.

2. Patterson-Kelley V-Type Blenders

   a. Transfer half of the feed to the blender shell. Load the blender with the two ports pointing upwards. Make sure the bottom port is sealed tightly before loading. Spread the feed evenly in the bottom of the blender. Add the premix in roughly equal portions to each port and spread it evenly over the feed. Add the remaing feed in a even layer over the premix. Seal the lids tightly on the top ports.

   b. Mix the diet in the blender for 15 minutes, using the intensifier bar only during the first 5 minutes.

E. Remove at least a 10 g sample from each diet. Part of the sample is for analysis and the remainder is for archival.

F. Transfer the rest of the diet to a plastic bag and

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

label it clearly with the study number, date, chemical, concentration and your initials. To aid in identification the bag may be color coded with tape for the group and sex.

Approved: 27 MAY88
(Date)

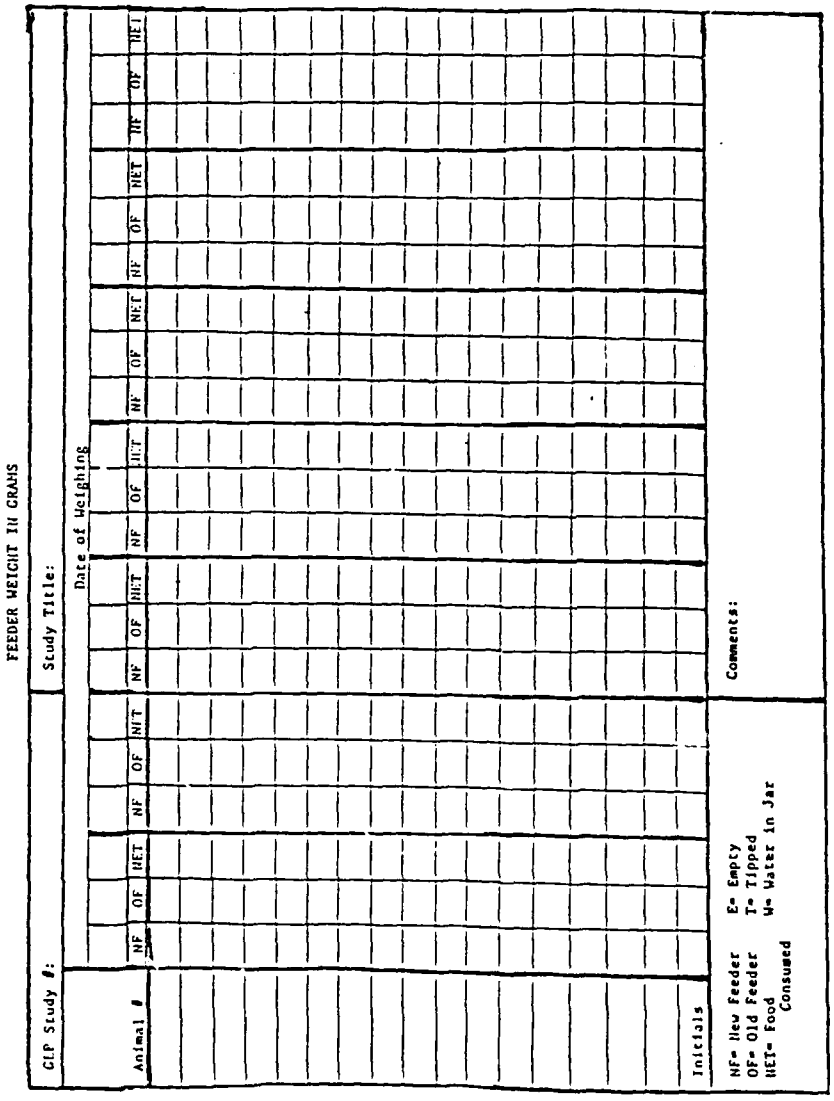
DON W. KORTE, JR., PhD
MAJ, MSC
Chief, Division of Toxicology

Certified: 27 May 88
(Date)

CAROLYN M. LEWIS, MS
Cheif, Quality Assurance

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

Figure 1



Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

Figure 2

GLP Study #_____ Date_____

CALCULATIONS FOR TARGET CONCENTRATION OF DIET

Group #_____ Dose Level_____mg/kg/day

Sex_____ For Dates_____to_____

1. Mean Daily Food Consumption (MDFC)

   = _____ g/day

2. Mean Body Weight (MBW)

   = _____ kg

3. Target Concentration (TC)

   = Dose level X MBW ÷ MDFC = _____ mg/g

4. Correction Factor (CF) - Optional

   = _____

5. Target Concentration Adjusted for Growth - Optional

   = CF X TC = _____ mg/g

Comments:

Prepared by_____

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

Figure 3

GLP Study #_____ Date_____

BLENDING OF A SINGLE PREMIX FOR ALL DIETS

1. Test Compound

   Lot No(s). (if available) _____

   _____

   Weight _____ g

   LAIR ID # of Balance Used _____

2. Feed

   Lot No(s). _____

   _____

   Weight _____g

   LAIR ID # of Balance Used _____

Comments:

Prepared by _____

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

Figure 4

GLP Study #__________ Date__________

BLENDING OF SEPARATE PREMIXES FOR EACH DIET

Lot No(s) of Test Chemical__________

____

Lot No(s). of Feed __________

____

LAIR ID No(s). of Balance(s) Used __________

____

Group #____ Sex____	Group #____ Sex____
Conc.____mg/g Batch #____	Conc.____mg/g Batch #____
Wt. of Chemical____g	Wt. of Chemical____g
Wt. of Feed____g	Wt. of Feed____g
Group #____ Sex____	Group #____ Sex____
Conc.____mg/g Batch #____	Conc.____mg/g Batch #____
Wt. of Chemical____g	Wt. of Chemical____g
Wt. of Feed____g	Wt. of Feed____g
Group #____ Sex____	Group #____ Sex____
Conc.____mg/g Batch #____	Conc.____mg/g Batch #____
Wt. of Chemical____g	Wt. of Chemical____g
Wt. of Feed____g	Wt. of Feed____g

Prepared by __________

Appendix E (cont.): PROCEDURES FOR DIET PREPARATION

Figure 5

GLP Study #______________ Date______________

BLENDING OF DIETS

Lot No(s). of Feed ______________________________

____

Date(s) Premix Prepared ______________________________

____

LAIR ID No(s). of Balance(s) Used ________________

____

Group #______ Sex______
Conc.________mg/g Batch #____
Wt. of Premix__________________g
Wt. of Feed__________________g
__________________g

Group #______ Sex______
Conc.________mg/g Batch #____
Wt. of Premix__________________g
Wt. of Feed__________________g
__________________g

Group #______ Sex______
Conc.________mg/g Batch #____
Wt. of Premix__________________g
Wt. of Feed__________________g
__________________g

Group #______ Sex______
Conc.________mg/g Batch #____
Wt. of Premix__________________g
Wt. of Feed__________________g
__________________g

Group #______ Sex______
Conc.________mg/g Batch #____
Wt. of Premix__________________g
Wt. of Feed__________________g
__________________g

Group #______ Sex______
Conc.________mg/g Batch #____
Wt. of Premix__________________g
Wt. of Feed__________________g
__________________g

Prepared by ________________

Appendix F: ANALYSIS OF FEED MIXTURES

Feed mixtures containing pyridostigmine bromide (PYR) were prepared for the 180-day portion of GLP Study 86005 to provide dose levels of 1 and 10 mg/kg body weight/day. New diets were prepared weekly to compensate for changes in food consumption and body weights due to growth. The target concentration of PYR in the feed mixtures ranged from 0.0114 to 0.2684 mg PYR/g feed.

Materials

Pyridostigmine bromide (Lot No. 590034) was supplied by Walter Reed Army Institute of Research (Washington, DC). Certified Rodent Chow #5002 (Lot Nos. JULY10861AMEAL, SEP03862AMEAL, SEP05862DMEAL, OCT03861EMEAL, JULY22861DMEAL, NOV13862CMEAL, NOV05861BMEAL, DEC04862EMEAL, JAN05871BMEAL, DEC16862DMEAL, FEB04872BMEAL, FEB26871DMEAL) was obtained from Ralston Purina (St. Louis, MO). All other chemicals were reagent grade. Tetramethylammonium chloride and 1-heptanesulfonic acid, sodium salt, were obtained from Aldrich Chemical Company (Milwaukee, WI); sodium phosphate monobasic was obtained from J.T. Baker Chemical Company (Phillipsburg, NJ). The water used in preparation of all HPLC solutions was deionized, distilled, and purified of organics using an Organicpure Water Purifier (Barnstead, Boston, MA).

The chromatographic system consisted of a Hewlett-Packard 1090 liquid chromatograph with diode array detector, an 85 B Personal Computer, a DPU Multichannel Integrator, and a ThinkJet Printer (Santa Clara, CA). Separations were obtained on a Brownlee silica column (4.6 x 100 mm, Brownlee Labs, Inc., Santa Clara, CA).

Methods

Stock solutions of PYR were made at two concentrations for use in the preparation of the standard curve. Solution 1 contained 10 mg PYR/ml water and Solution 2 contained 1 mg PYR/ml water. Each solution was divided into 500-µl portions, placed in plastic microcentrifuge tubes and stored in the freezer. New stock solutions were made every month. Six concentrations of PYR in rodent chow were used for the standard curve and were prepared by adding various amounts of the stock solutions to rodent chow as shown in Table 1.

Appendix F (cont.): ANALYSIS OF FEED MIXTURES

Table 1

Level	Target Conc. (mg PYR/g chow)	Chow (g)	Amt. of Stock Solution (µl)	Stock Solution #
1	2.00	1	200	1
2	1.00	1	100	1
3	0.50	1	50	1
4	0.10	1	100	2
5	0.05	1	50	2
6	0.01	2	20	2

A standard curve was run every day that analyses were performed.

Samples of the feed mixtures were extracted for analysis by adding water, shaking on a mechanical shaker, and centrifuging. The supernatant was poured into a volumetric flask, based on the concentration of sample as shown in Table 2.

Table 2

Dose Level (mg PYR/ g chow)	g of Diet Analyzed	ml of Water Added per Extraction	Minutes of Shaking per Extraction	Number of Extractions per Sample	Final Vol. (ml)
2.00	1	35	15	4	200
1.00	1	35	15	4	200
0.50	1	35	15	4	200
0.10	1	25	40	2	50
0.05	1	25	40	2	50
0.01	2	25	40	2	50

The volumetrics containing the combined extracts of each sample were brought to volume with water and mixed well.

Appendix F (cont.): ANALYSIS OF FEED MIXTURES

Small portions of these solutions were filtered through 0.2 µm membrane filters directly into sample vials for subsequent HPLC analysis.

To determine the homogeneity of the feed mixtures, samples were taken from the left, right, and bottom ports of the Twin Shell Blender used in the preparation of the diet and analyzed in duplicate or triplicate for each dose level. Samples for testing homogeneity were collected during the first and thirteenth weeks of the study.

The analysis of PYR in the feed mixtures was accomplished under the following HPLC conditions:

Column: Brownlee silica 5 µm (100 x 4.6 mm)
Flow: 1.5 ml/min
Mobile Phase: 20% acetonitrile, 80% buffer
Buffer: 0.01 M heptanesulfonic acid
0.01 M sodium dihydrogen phosphate
0.0025 M tetramethylammonium chloride
pH adjusted to 3 with sulfuric acid
Wavelength Monitored: 269 nm
Injection Volume: 25 µl

Under these conditions, PYR eluted with a retention time of 2.5 minutes.

Calculations

All calculations were performed on either a TI 55-111 calculator or the HP-85 personal computer, which is part of the HP 1090 HPLC System. Results were in close agreement using either method. Least squares linear regression analysis of the standard concentration versus the peak height of PYR was performed to obtain the equation of the best fitting line in the form of

$$y = mx + b$$

where y is the peak height, m is the slope, x is the concentration (ng/µl), and b is the intercept. The concentration of each sample was calculated by substituting for y the peak height obtained by HPLC analysis and solving for x. To calculate the concentration of PYR in the diet in terms of mg PYR/g diet, the concentration of extract was multiplied by the dilution factor and divided by the weight of the diet sample extracted.

Appendix F (cont.): ANALYSIS OF FEED MIXTURES

$$\text{Concentration in diet} = \frac{\text{Conc. of extract x dilution factor}}{\text{Grams of diet extracted}}$$

When the calculations were performed on the 85 B personal computer, an average standard was entered into the program and the points on the standard curve run each day were averaged in with this curve. The resulting standard curve was used for calculating the values for that day's samples. Final concentrations of PYR in the diet were calculated on the 85 B by entering the proper dilution factor for each sample before the runs were made. All calculations were performed when the runs were integrated and the results were printed out on the chromatographic reports.

Initial intentions were to use the 85 B for all calculations since it was more convenient and less time consuming than the TI 55-111. However, due to either operator or instrumental error, results were not always obtained from the 85 B. In these instances, the TI 55-111 was used.

After the first month of the study, it was noted that the results for the lowest concentration dose were more consistent and accurate when a standard curve consisting of only the lowest three values of the daily standard curve was used. This curve was always calculated using the TI 55-111.

The plots of PYR concentration versus the peak height were linear within the range of concentrations analyzed. The results of the regression analysis for each run and the method of calculation are shown in Table 3.

Table 3: Regression Analysis Values for Each Run

Date of Run	y-intercept	Slope	Method of Calculation
5-Nov-86*	----	----	85 B
6-Nov-86	0.11991	0.06701	TI 55-111
7-Nov-86	-0.11140	0.07134	TI 55-111
10-Nov-86	-0.09979	0.07240	TI 55-111
12-Nov-86*	----	----	85 B
13-Nov-86	-0.13481	0.07124	TI 55-111

* These results were not printed out and saved.

Appendix F (cont.): ANALYSIS OF FEED MIXTURES

Table 3 (cont.): Regression Analysis Values for Each Run

Date of Run	y-intercept	Slope	Method of Calculation
14-Nov-86	0.21714	0.06763	TI 55-111
17-Nov-86	-0.15374	0.07350	TI 55-111
18-Nov-86*	----	----	85 B
19-Nov-86	-0.02409	0.06918	TI 55-111
20-Nov-86	-0.02409	0.06918	TI 55-111
21-Nov-86	0.02978	0.06992	85 B
24-Nov-86	-0.10483	0.07487	85 B
25-Nov-86	-0.10483	0.07487	85 B
1-Dec-86	0.04999	0.06381**	85 B
2-Dec-86	-0.11485	0.06261	85 B
3-Dec-86	0.18716	0.05320	TI 55-111
3-Dec-86 (low conc)	-0.07324	0.05895	TI 55-111
4-Dec-86	-0.02315	0.06153	TI 55-111
4-Dec-86 (low conc)	-0.05328	0.06137	TI 55-111
10-Dec-86	0.04529	0.06122	85 B
11-Dec-86	-0.03430	0.06159	85 B
12-Dec-86	-0.03430	0.06160	85 B
17-Dec-86	0.04519	0.06360	85 B
18-Dec-86	0.06507	0.06427	85 B
5-Jan-87	0.09999	0.06428	85 B
6-Jan-87	0.04261	0.06681	85 B
6-Jan-87 (low conc)	-0.02199	0.06834	TI 55-111
7-Jan-87	0.03631	0.06719	85 B
7-Jan-87 (low conc)	0.08869	0.07162	TI 55-111
9-Jan-87	0.04124	0.06607	85 B
9-Jan-87 (low conc)	-0.06343	0.06929	TI 55-111
12-Jan-87	0.03472	0.06506	85 B
13-Jan-87	0.05315	0.06639	85 B
14-Jan-87	-0.02611	0.06859	85 B
15-Jan-87	-0.03996	0.06839	85 B
20-Jan-87	0.00972	0.06744	85 B
21-Jan-87	-0.03440	0.06749	85 B
22-Jan-87	-0.00037	0.06749	85 B
27-Jan-87	-0.01826	0.06223**	85 B
28-Jan-87	-0.01548	0.06419	TI 55-111
29-Jan-87	-0.10175	0.06136	85 B
2-Feb-87	-0.00757	0.06179	85 B

* These results were not printed out and saved.
** The column went dry and affected the slope of the standard curve.

Appendix F (cont.): ANALYSIS OF FEED MIXTURES

Table 3 (cont.): Regression Analysis Values for Each Run

Date of Run	y-intercept	Slope	Method of Calculation
3-Feb-87	-0.00757	0.06179	85 B
3-Feb-87 (low conc)	-0.03661	0.06215	TI 55-111
4-Feb-87	-0.04286	0.06288	85 B
5-Feb-87	-0.03285	0.06224	85 B
9-Feb-87	-0.01013	0.06192	85 B
9-Feb-87 (low conc)	-0.09660	0.06329	TI 55-111
11-Feb-87	-0.02811	0.06218	85 B
11-Feb-87 (low conc)	-0.07611	0.06165	TI 55-111
12-Feb-87	-0.02435	0.06189	85 B
12-Feb-87 (low conc)	-0.06025	0.06128	TI 55-111
18-Feb-87	-0.01429	0.06154	85 B
18-Feb-87 (low conc)	-0.06049	0.06064	TI 55-111
19-Feb-87	-0.08454	0.06434	85 B
25-Feb-87	0.09787	0.05144*	85 B
25-Feb-87 (low conc)	-0.06000	0.05496	TI 55-111
26-Feb-87	-0.05239	0.04827	85 B
4-Mar-87	0.02750	0.05762	85 B
5-Mar-87	0.00086	0.05842	85 B
11-Mar-87	0.05947	0.05599	85 B
12-Mar-87	0.00224	0.05686	85 B
18-Mar-87	0.03156	0.05721	85 B
18-Mar-87 (low conc)	-0.03014	0.05723	TI 55-111
25-Mar-87	-0.00694	0.05624	85 B
25-Mar-87 (low conc)	0.03689	0.05099	TI 55-111
1-Apr-87	0.00634	0.05629	85 B
8-Apr-87	-0.02953	0.05749	85 B
8-Apr-87 (low conc)	0.03247	0.05749	TI 55-111
21-Apr-87	0.02658	0.06218	85 B
22-Apr-87	-0.03619	0.05784	85 B
5-May-87	-0.01189	0.05723	85 B
11-May-87	-0.06157	0.05754	85 B
11-May-87 (low conc)	-0.07033	0.05509	TI 55-111

* The column was replaced with a new one.

The results from the analysis of the diet mixtures are shown in Table 4.

Appendix F (cont.): ANALYSIS OF FEED MIXTURES

Table 4

Group	Study Week	Target Conc.*	Batch Number	Date Prepared	Date Analyzed	Conc. Determined by Analysis	% of Target Conc.
2	1	0.0114	1	4-Nov-86	5-Nov-86	0.0122	107.0
7,7A	1	0.1071	1	4-Nov-86	10-Nov-86	0.0991	92.5
7,7A	1	0.1071	2	4-Nov-86	7-Nov-86	0.0997	93.2
3,3A	1	0.1064	1	4-Nov-86	10-Nov-86	0.0972	91.4
3,3A	1	0.1064	2	4-Nov-86	10-Nov-86	0.0989	93.0
7,7A	2	0.1110	1	10-Nov-86	12-Nov-86	0.1015	91.4
7,7A	2	0.1110	2	10-Nov-86	13-Nov-86	0.1020	91.9
2	2	0.0124	1	11-Nov-86	17-Nov-86	0.0127	102.4
2	2	0.0124	2	11-Nov-86	17-Nov-86	0.0131	105.6
3,3A	2	0.1190	1	11-Nov-86	13-Nov-86	0.1092	91.8
3,3A	2	0.1190	2	11-Nov-86	17-Nov-86	0.1109	93.2
7,7A	3	0.1225	1	17-Nov-86	20-Nov-86	0.1126	91.9
7,7A	3	0.1225	2	17-Nov-86	20-Nov-86	0.1134	92.6
2	3	0.0135	1	18-Nov-86	25-Nov-86	0.0122	90.4
2	3	0.0135	2	18-Nov-86	25-Nov-86	0.0126	93.3
3,3A	3	0.1340	1	18-Nov-86	19-Nov-86	0.1220	91.0
3,3A	3	0.1340	2	18-Nov-86	19-Nov-86	0.1180	88.1
7,7A	4	0.1370	1	24-Nov-86	25-Nov-86	0.1250	91.2
7,7A	4	0.1370	2	24-Nov-86	25-Nov-86	0.1230	89.8
2	4	0.0150	1	25-Nov-86	2-Dec-86	0.0138	92.0
2	4	0.0150	2	25-Nov-86	2-Dec-86	0.0138	92.0
3,3A	4	0.1470	1	25-Nov-86	2-Dec-86	0.1327	90.3
3,3A	4	0.1470	2	25-Nov-86	2-Dec-86	0.1362	92.7
7,7A	5	0.1520	1	1-Dec-86	3-Dec-86	0.1505	99.0
7,7A	5	0.1520	2	1-Dec-86	3-Dec-86	0.1496	98.4
2	5	0.0158	1	2-Dec-86	3-Dec-86	0.0146	92.4
2	5	0.0158	2	2-Dec-86	3-Dec-86	0.0144	91.1
3,3A	5	0.1520	1	2-Dec-86	4-Dec-86	0.1370	90.1
3,3A	5	0.1520	2	2-Dec-86	4-Dec-86	0.1320	86.8
7,7A	6	0.1715	1	8-Dec-86	10-Dec-86	0.1624	94.7
7,7A	6	0.1715	2	8-Dec-86	10-Dec-86	0.1660	96.8
2	6	0.0172	1	9-Dec-86	10-Dec-86	0.0185	107.6
2	6	0.0172	2	9-Dec-86	10-Dec-86	0.0177	102.9
3,3A	6	0.1702	1	9-Dec-86	12-Dec-86	0.1677	98.5
3,3A	6	0.1702	2	9-Dec-86	12-Dec-86	0.1756	103.2
7,7A	7	0.1770	1	15-Dec-86	17-Dec-86	0.1840	104.0
7,7A	7	0.1770	2	15-Dec-86	17-Dec-86	0.1860	105.1

* mg PYR/g chow.

Appendix F (cont.): ANALYSIS OF FEED MIXTURES

Table 4 (cont.)

Group	Study Week	Target Conc.*	Batch Number	Date Prepared	Date Analyzed	Conc. Determined by Analysis	% of Target Conc.
2	7	0.0183	1	16-Dec-86	9-Jan-87	0.0180	98.4
2	7	0.0183	2	16-Dec-86	9-Jan-87	0.0165	90.2
3,3A	7	0.1763	1	16-Dec-86	17-Dec-86	0.1843	104.5
3,3A	7	0.1763	2	16-Dec-86	17-Dec-86	0.1869	106.0
7,7A	8	0.1746	1	22-Dec-86	5-Jan-87	0.1659	95.0
7,7A	8	0.1746	2	22-Dec-86	5-Jan-87	0.1725	98.8
2	8	0.0180	1	23-Dec-86	9-Jan-87	0.0156	86.7
2	8	0.0180	2	23-Dec-86	9-Jan-87	0.0163	90.6
3,3A	8	0.1841	1	23-Dec-86	9-Jan-87	0.1759	95.5
3,3A	8	0.1841	2	23-Dec-86	9-Jan-87	0.1809	98.3
7,7A	9	0.1798	1	29-Dec-86	7-Jan-87	0.1782	99.1
7,7A	9	0.1798	2	29-Dec-86	7-Jan-87	0.1710	95.1
2	9	0.0188	1	30-Dec-86	12-Jan-87	0.0171	91.0
2	9	0.0188	2	30-Dec-86	12-Jan-87	0.0166	88.3
3,3A	9	0.1831	1	30-Dec-86	7-Jan-87	0.1781	97.3
3,3A	9	0.1831	2	30-Dec-86	7-Jan-87	0.1768	96.6
7,7A	10	0.1846	1	5-Jan-87	13-Jan-87	0.1821	98.6
7,7A	10	0.1846	2	5-Jan-87	13-Jan-87	0.1727	93.6
2	10	0.0199	1	6-Jan-87	14-Jan-87	0.0180	90.5
2	10	0.0199	2	6-Jan-87	14-Jan-87	0.0172	86.4
3,3A	10	0.1939	1	6-Jan-87	13-Jan-87	0.1778	91.7
3,3A	10	0.1939	2	6-Jan-87	13-Jan-87	0.1838	94.8
7,7A	11	0.1962	1	12-Jan-87	15-Jan-87	0.1952	99.5
7,7A	11	0.1962	2	12-Jan-87	15-Jan-87	0.1719	87.6
2	11	0.0200	1	13-Jan-87	20-Jan-87	0.0182	91.0
2	11	0.0200	2	13-Jan-87	20-Jan-87	0.0194	97.0
3,3A	11	0.2005	1	13-Jan-87	15-Jan-87	0.1930	96.3
3,3A	11	0.2005	2	13-Jan-87	15-Jan-87	0.1969	98.2
7,7A	12	0.2024	1	19-Jan-87	21-Jan-87	0.2004	99.0
7,7A	12	0.2024	2	19-Jan-87	21-Jan-87	0.1987	98.2
2	12	0.0215	1	20-Jan-87	22-Jan-87	0.0188	87.4
2	12	0.0215	2	20-Jan-87	22-Jan-87	0.0185	86.0
3,3A	12	0.2045	1	20-Jan-87	21-Jan-87	0.1976	96.6
3,3A	12	0.2045	2	20-Jan-87	21-Jan-87	0.2055	100.5
7,7A	13	0.2375	1	26-Jan-87	28-Jan-87	0.2286	96.3
7,7A	13	0.2375	2	26-Jan-87	28-Jan-87	0.2294	96.6
2	13	0.0237	1	27-Jan-87	29-Jan-87	0.0227	95.8

* mg PYR/g chow.

Appendix F (cont.): ANALYSIS OF FEED MIXTURES

Table 4 (cont.)

Group	Study Week	Target Conc.*	Batch Number	Date Prepared	Date Analyzed	Conc. Determined by Analysis	% of Target Conc.
2	13	0.0237	2	27-Jan-87	29-Jan-87	0.0225	94.9
3,3A	13	0.2208	1	27-Jan-87	2-Feb-87	0.2183	98.9
3,3A	13	0.2208	2	27-Jan-87	2-Feb-87	0.2182	98.8
7,7A	14	0.2124	1	2-Feb-87	5-Feb-87	0.2145	101.0
7,7A	14	0.2124	2	2-Feb-87	5-Feb-87	0.2107	99.2
2	14	0.0229	1	3-Feb-87	4-Feb-87	0.0204	89.1
3,3A	14	0.2183	1	3-Feb-87	5-Feb-87	0.2096	96.0
3,3A	14	0.2183	2	3-Feb-87	5-Feb-87	0.2023	92.7
7,7A	15	0.2039	1	9-Feb-87	11-Feb-87	0.1991	97.6
2	15	0.0229	1	10-Feb-87	11-Feb-87	0.0217	94.8
3,3A	15	0.2323	1	10-Feb-87	11-Feb-87	0.2195	94.5
2	16	0.0235	1	17-Feb-87	18-Feb-87	0.0219	93.2
3,3A	16	0.2292	1	17-Feb-87	18-Feb-87	0.2198	95.9
7,7A	16	0.2546	1	17-Feb-87	18-Feb-87	0.2395	94.1
2	17	0.0238	1	24-Feb-87	25-Feb-87	0.0215	90.3
7,7A	17	0.2393	1	24-Feb-87	25-Feb-87	0.2484	103.8
3,3A	17	0.2684	1	24-Feb-87	25-Feb-87	0.2484	92.5
2	18	0.0242	1	3-Mar-87	4-Mar-87	0.0227	93.8
3,3A	18	0.2446	1	3-Mar-87	4-Mar-87	0.2380	97.3
7,7A	18	0.2477	1	3-Mar-87	4-Mar-87	0.2520	101.7
2	19	0.0242	1	10-Mar-87	11-Mar-87	0.0233	96.3
7,7A	19	0.2314	1	10-Mar-87	11-Mar-87	0.2429	105.0
3,3A	19	0.2430	1	10-Mar-87	11-Mar-87	0.2291	94.3
2	20	0.0246	1	17-Mar-87	18-Mar-87	0.0228	92.7
7,7A	20	0.2376	1	17-Mar-87	18-Mar-87	0.2399	101.0
3,3A	20	0.2487	1	17-Mar-87	18-Mar-87	0.2472	99.4
2	21	0.0241	1	24-Mar-87	25-Mar-87	0.0239	99.2
3,3A	21	0.2357	1	24-Mar-87	25-Mar-87	0.2375	100.8
7,7A	21	0.2393	1	24-Mar-87	25-Mar-87	0.2590	108.2
2	22	0.0232	1	31-Mar-87	1-Apr-87	0.0232	100.0
3,3A	22	0.2434	1	31-Mar-87	1-Apr-87	0.2487	102.2
7,7A	22	0.2527	1	31-Mar-87	1-Apr-87	0.2621	103.7
2	23	0.0256	1	7-Apr-87	8-Apr-87	0.0251	98.0
3,3A	23	0.2508	1	7-Apr-87	8-Apr-87	0.2235	89.1
7,7A	23	0.2515	1	7-Apr-87	8-Apr-87	0.2576	102.4
2	24	0.0256	1	14-Apr-87	21-Apr-87	0.0251	98.0
7,7A	24	0.2510	1	14-Apr-87	21-Apr-87	0.2606	103.8
3,3A	24	0.2504	1	14-Apr-87	21-Apr-87	0.2411	96.3
2	25	0.0248	1	21-Apr-87	22-Apr-87	0.0275	110.9

* mg PYR/g chow.

Appendix F (cont.): ANALYSIS OF FEED MIXTURES

Table 4 (cont.)

Group	Study Week	Target Conc.*	Batch Number	Date Prepared	Date Analyzed	Conc. Determined by Analysis	% of Target Conc.
3,3A	25	0.2553	1	21-Apr-87	22-Apr-87	0.2575	100.9
7,7A	25	0.2599	1	21-Apr-87	22-Apr-87	0.2569	98.8
2	26	0.0323	1	28-Apr-87	5-May-87	0.0298	92.3
3,3A	26	0.2506	1	28-Apr-87	5-May-87	0.2536	101.2
7,7A	26	0.2553	1	28-Apr-87	5-May-87	0.2444	95.7
2	27	0.0261	1	5-May-87	11-May-87	0.0234	89.7
3,3A	27	0.2622	1	5-May-87	11-May-87	0.2791	106.4

* mg PYR/g chow.

Results of the homogeneity study are presented in Tables 5 and 6.

Table 5

Target Conc. of PYR (mg/g)	Site of Sampling	Conc. Detn. by Analysis (mg/g)	Mean Conc. (mg/g)	Absolute Deviation from Mean (%)
		Week 1		
	Right	0.01208		0.8
0.0114	Left	0.01220	0.01218	0.2
	Bottom	0.01225		0.6
	Right	0.09357		2.5
0.1070	Left	0.09537	0.09596	0.6
	Bottom	0.09893		3.1
	Right	0.3256		3.4
0.3260	Left	0.3046	0.3149	3.3
	Bottom	0.3145		0.1
	Right	0.6140		0.2
0.6339	Left	0.6272	0.6153	1.9
	Bottom	0.6048		1.7
	Right	1.0461		5.4
1.0099	Left	1.0136	0.9927	2.1
	Bottom	0.9185		7.5

Appendix F (cont.): ANALYSIS OF FEED MIXTURES

Table 6

Target Conc. of PYR (mg/g)	Site of Sampling	Conc. Detn. by Analysis (mg/g)	Mean Conc. (mg/g)	Absolute Deviation from Mean (%)
		Week 13		
0.0237	Right	0.0227	0.0224	1.2
	Left	0.0227		1.2
	Bottom	0.0219		2.4
0.2375	Right	0.2292	0.2255	1.6
	Left	0.2286		1.4
	Bottom	0.2187		3.0
0.2208	Right	0.2183	0.2185	0.1
	Left	0.2234		2.2
	Bottom	0.2138		2.2
0.6722	Right	0.6519	0.6550	0.5
	Left	0.6643		1.4
	Bottom	0.6489		0.9
1.3443	Right	1.3841	1.4073	1.7
	Left	1.4261		1.3
	Bottom	1.4118		0.3
2.5908	Right	2.7393	2.7413	0.1
	Left	2.7495		0.3
	Bottom	2.7352		0.2

Discussion

The concentration of PYR in the mixtures was within 10% of the target concentration with the exception of 11% of the diet mixture samples, which were within 10-15% of the target concentration. Samples collected during the first and thirteenth weeks of the study showed that the PYR concentrations were homogeneous in the feed over the range tested, according to the EPA and NIH criteria for homogeneity[1].

[1] EPA, GLP Standards, Final Rule (40 CFR 160) as published in the Federal Register, Vol. 48, n.1. 230, Nov 29, 1983, p. 53955-53959.

Appendix G: PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

Title: AutoAnalyzer II Procedure for the Determination of Erythrocyte Acetylcholinesterase and Plasma Cholinesterase Activities in Pyridostigmine - Inhibited Blood.

Scope: This SOP specifies the instrumentation, reagents, and procedures used to measure cholinesterase activities in animal blood derived from investigations which involve the use of pyridostigmine or similar anti-cholinesterase compounds.

References:

1. FDA GLP regulations (21 CFR58) and preamble as published in the Federal Register, 22 Aug 78 (43 FR 5986-60025).

2. EPA GLP regulations (40 CFR792) and preamble as published in the Federal Register, 29 Nov 83 (48 FR 53922).

3. Kaminskis, A., "Determination of Erythrocyte Acetylcholinesterase Activity in Pyridostigmine Inhibited Human Blood." SOP Analytical Chemistry Branch, US Army Medical Research Institute for Chemical Defense, 18 Jun 85.

4. Ellman, G.L., K.D. Courtney, V. Andres, Jr. and R.M. Featherstone. "A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity." Biochem. Pharm. 7:88-95, 1961.

5. Humiston, C.G. and G.J. Wright. "An Automated Method for the Determination of Cholinesterase Activity." Toxicology and Applied Pharm. 10:467-480, 1967.

6. Groff, W.A., A. Kaminskis and R.I. Ellin. "Interconversion of Cholinesterase Enzyme Activity Units by the Manual Delta pH Method and a Recommended Automated Method." Clin Tox.. 9:353-358, 1976.

7. Technical Publication No. TG1-0170-01, "Course Guide for the Technicon AutoAnalyzer II System." Technicon Instruments Corp., Tarrytown, New York, Aug 72.

8. Technicon Manual No. TP1-0170-10, "Programmed Instruction for the Technicon AutoAnalyzer II System." Technicon Instruments Corp., Tarrytown, New York, Dec 73.

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

9. Meyer, S.L. Straight-line Graphs and Fitting. In: Data Analysis for Scientists and Engineers. John Wiley & Sons, Inc., New York, NY, 1975, pp 71-75.

Purpose: To apply the essential requirements of an accepted method for measuring cholinesterase activity in human blood (3) in performing comparable measurements using animal blood.

Justification for Reference Method Modifications

Species differences in erythrocyte and plasma cholinesterase activities prohibit direct utilization of previously reported methods without some modification. To establish comparability of values between human and animal blood, the reference method was modified to increase measurement sensitivity at lower activity levels and to compensate for species variability in hemolytic susceptibility. The basic reaction mechanism and underlying measurement principles were not changed. A list of hardware and procedural differences is included in Appendix A.

Method Derivation and Reaction Mechanism

This SOP was adapted from the AutoAnalyzer procedure of Kaminskis for measuring acetylcholinesterase activity in human erythrocytes (3). His method was based on the basic reaction mechanism of Ellman's manual assay (4) as previously modified for semi-automated continuous flow analyses (5,6).

In the presence of nonlimiting amounts of acetylthiocholine substrate under controlled reaction conditions, red cell acetylcholinesterase (E.C. 3.1.1.7) and plasma cholinesterase (E.C. 3.1.1.8) catalyze the production of thiocholine and acetic acid at rates proportionate to enzyme concentration. Thiocholine reacts with DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid), to produce equimolar amounts of a mixed disulfide and colored dianion, 2-nitro-5-thiobenzoic acid. The absorbance change at 410 nm occurring within a measured time period is proportionate to enzymatic activity when properly blanked and calibrated.

Equipment and Materials

A. Instruments

1. AutoAnalyzer II System consisting of a Technicon Sampler IV, Proportioning Pump III, Two Channel Recorder, two Single

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

Channel Colorimeters with 15 mm flow cells and 410 nm filters, and two identical Cholinesterase Chemistry Modules constructed according to the manifold diagram shown in Fig. 1 using the hardware components listed in Appendix A.

2. Eppendorf Micro-Centrifuge Model 5412.

3. Beckman Altex PHI 61 pH meter.

B. **Miscellaneous Equipment**

1. Analytical balance

2. Eppendorf and Gilsen pipettors and disposable tips

3. 1.5 mL polypropylene centrifuge tubes

4. 2.5 mL AutoAnalyzer cups

5. 0-100 linear chart scale paper

C. Chemicals

Chemical Name	Supplier's Address	Catalog #
Tris (hydroxymethyl) aminomethane	Sigma Chemical Co St Louis, MO	T-1503
5,5'-dithiobis (2-nitrobenzoic acid)	. . . . .	D-8130
Brij 35,30% solution	. . . . .	430 AG-6
Acetylthiocholine iodide	. . . . .	A-5751
Reduced Glutathione	. . . . .	G-4251
Eel Acetylcholinesterase, Type VI-S	. . . . .	C-3389
Bovine Albumin, Fx V	. . . . .	A-4503
Hydrochloric Acid	JT Baker Chemicals Co Phillispsburg, NJ	9530-3
Sodium Chloride (NaCl)AR	. . . . .	5-3624
Ethylenedinitrilo-tetraacetic acid (EDTA) disodium dihydrate	Mallinchrodt Chemical Works, St. Louis, MO	4931

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

AutoAnalyzer System Mechanisms

The system Flow Diagram is shown in Figure 1. Sampled specimens are split into two equal aliquots which are injected into separate but identical flow pathways. One path combines DTNB reagent (Tris-buffered at pH 8.2) with specimen and acetylthiocholine in saline (Channel A). The second substitutes saline without substrate in an otherwise identical blanking reaction mixture (Channel B). Both streams are incubated in 37° C heating baths before passage through 24 inch dialyzers.

Dialysates in Tris buffer are mixed and debubbled prior to passage through the flow cells of two colorimeters. A 30/hr, 1:2 (sample: wash) cam in the Sampler IV provides acceptable flow cell flushing for baseline recovery between peaks.

Procedures

Preparation of Reagents

1) **AutoAnalyzer Wash Solution:**

Add 1.5 mL of Brij 35, 30% solution to 1 L of distilled deionized water (DD). Mix thoroughly with magnetic stirrer.

2) **50 mM Tris Buffer, pH 8.2, containing 114 mM NaCl:**

Dissolve 6.05 g Tris base and 6.64 g NaCl in 900 mL DD-water. Adjust pH to 8.2 by drop wise addition of con HCL. Dilute to 1L with DD water and add 1.5 mL of Brij 35. Confirm pH after thorough mixing and readjust if necessary.

3) **1.68 mM DTNB reagent:**

Dissolve 0.6653 g DTNB in 1 L of 50 mM Tris buffer, pH 8.2 (see above). Mix with magnetic stirrer until clear, yellow solution. Confirm pH 8.2 and adjust if necessary.

4) **0.9% NaCl:**

Dissolve 9 g NaCl in 900 mL DD water and dilute to 1 L with DD water.

5) **1mM EDTA:**

Dissolve 0.372 g EDTA-disodium dihydrate salt in 1 L DD water.

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

6) **12.7 mM Acetylthiocholine (ATC) Substrate:**

Prepare 100 mM ATC by dissolving 1.4478g of acetylthiocholine iodide in 50 mL of 0.9% saline. Dilute 12.7 mL of 100 mM ATC to 100 mL using 0.9% saline.

Preparation of Standards and Controls

1) **60 mM Reduced Glutathione (GSH) Stock Standard:**

Dissolve 0.9219 g GSH in 40 mL of 1 mM EDTA. Dilute to 50 mL with 1 mM EDTA. Store in refrigerator in separate 1 mL aliquots in tightly capped vials. Stock preparation is stable up to 6 months at refrigerator temperature.

2) **Working GSH Standard Dilutions:**

On day of assay, warm an aliquot of 60 mM GSH stock standard. Add 0.2 mL of stock to 9.8 mL of 1 mM EDTA, and mix on vortex. Prepare standard dilutions according to the following table:

Lab #	Aliquot 60 mM GSH (mL)	+ Aliquot 1 mM EDTA (mL)	Concentration (umol GSH/mL)
S0	0	2.00	0
S1	0.25	1.75	0.15
S2	0.50	1.50	0.30
S3	1.00	1.00	0.60
S4	1.50	0.50	0.90
S5	2.00	0	1.20
S6	2.00	0	1.20

Prepare fresh dilutions for each day's assays and confirm concentrations using a spectrophotometric assay.

3) **Stock Eel Acetylcholinesterase Control:**

Dissolve 1 g Serum Bovine Albumin in 100 mL of 0.9% NaCl. Use 50 mL of this diluent to dissolve 1.7 mg of Eel Cholinesterase lyophilized powder. Store frozen in 1 mL aliquots in capped polypropylene tubes.

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

4) **Working Eel Acetylcholinesterase control dilutions:**

On day of assay, thaw an aliquot of stock control, and warm to room temperature. Prepare dilutions according to the following table:

Lab #	Aliquot stock control (mL)	+	aliquot saline diluent (mL)
E1	0.025		1.975
E2	0.050		1.950
E3	0.075		1.925
E4	0.100		1.900

Prepare fresh dilutions for each day's assays and include analyses of all dilutions in the beginning and end of each day's run.

Preparation of Blood Specimens

1) Use freshly drawn whole blood anticoagulated with EDTA.

2) Transfer aliquots into capillary tubes for duplicate Micro-hematocrit determinations on each specimen.

3) Transfer measured volumes of whole blood into 1.5 mL polypropylene centrifuge tubes labelled to identify specimens in the sample preparations shown below.

4) Centrifuge at 15000 RPM in Eppendorf centrifuge for 2 min.

5) Withdraw plasma as completely as possible without disturbing the packed red cells (PCV) and transfer plasma into I.D. labelled tubes.

6) Select one or more of the following options for red blood cell preparation:

a. **Unwashed intact red cells** - Add 1 mL 0.9% saline to PCV, gently mix to complete and uniform suspension, and transfer to AAII cups for immediate sampling.

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

b. **Washed intact red cells** - Perform saline suspension as for "unwashed" cells but remove and discard supernatants after 1 min centrifugation. Repeat twice before transferring final suspension into AAII cups for immediate sampling.

c. **Unwashed hemolyzed red cells** - Add measured volume of specified lysing solution (max 1.5 mL) to PCV after plasma removal. Mix on vortex mixer for 30 sec. Transfer to AAII cups for sampling.

d. **Washed hemolyzed red cells** - Perform saline washes as for "washed intact red cells" and discard supernatant after final wash. Continue preparation as for "unwashed hemolyzed red cells." Transfer to AAII cups for sampling.

7) Add measured volume of plasma from each specimen into a specified volume of 0.9% saline in identification labelled AAII cups and mix thoroughly before sampling by system.

8) Record sample preparation designation codes and note any exceptions for individual specimens for subsequent calculation of dilution factors.

Operation and Maintenance of AutoAnalyzer II System

1) Perform general maintenance operations as outlined in LAIR OP-ACH-26 except use Brij 35/water flushing solution.

2) Install or confirm correct chemistry module and pump tube manifold for assay shown in Flow Diagram of Figure 1.

3) Turn on power to all instrument modules for minimum 30 min warm up.

4) Engage pump tubes, insert platen and initiate flush of flow system using freshly prepared Brij 35/H_2O wash solution during warm up period. Observe bubble pattern for regularity of size and flow. Initiate corrective action according to OP-ACH-26 if required.

5) After stabilization of electronic components, check alignment of colorimeter signal ouputs with recorder scale. Use screwdriver adjustments on colorimeter as necessary: Display rotary switch position zero, recorder baseline, zero. Display rotary switch position full scale, recorder pen deflection full scale (100 chart units).

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

6) Set baseline controls to rotation midpoint (5 turns from either limit), all apertures fully open (aperture knurled screws rotated fully clockwise), and Std Cal controls of both channels to 350.

7) Initiate flow of reagents in pump tubes as shown in Figure 1. After 15 min, turn rotary display switches to position Normal on both colorimeters.

8) Set recorder pen positions for baseline (0 chart units) by adjusting reference apertures on both colorimeters. Use baseline controls for fine tuning.

Assay Calibration (7,8)

1) Activate Sampler to initiate sampling of GSH Std dilutions in the following sequence:

(1) 1.2 umol GSH/mL
(2) 1 mM EDTA blank
(3) 0.15 umol GSH/mL
(4) 0.30 " "
(5) 0.60 " "
(6) 0.90 " "
(7) 1.20 " "
(8) 1 mM EDTA blank.

2) Measure and record the reaction time in minutes from the point of substrate injection into the stream flow to dialyzer exit. (Additional air bubbles drawn into the stream during sampler probe movement from reservoir to sample cup and the color intensity of the highest GSH std can be used to perform this measurement accurately and reproducibly.) Reaction time approximates 3.9 min with the chemistry module components and pump tubes shown in Figure 1 and listed in Appendix A.

3) As the peak corresponding with the highest GSH std in the presence of ATC substrate is recorded, adjust the STD Cal control of the channel without substrate to achieve equivalent pen deflection. Record the control settings.

4) Observe peaks for succeeding series of GSH Std dilutions and repeat calibration procedure if equivalent response in the presence or absence of substrate is not confirmed throughout the std concentration range.

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

5) Set rotary display switches of both colorimeters to DAMP 1 position after acceptable calibration.

6) Assay a series of eel acetylcholinesterase Control dilutions immediately following a complete set of GSH Stds prior to and on completion of a designated group of specimen analyses.

7) Use GSH stds as markers during extended runs to confirm calibration stability and aid in peak identification.

Specimen Analyses

1) Initiate sampling of specimen preparations in a recorded sequence to enable peak identification on the chart record.

2) Repeat analysis of specimens exhibiting overscale chart peaks using appropriate dilutions of the cup preparation. Specify corrective action on the chart record at associated peak.

3) Repeat sampling of cup preparations whose peaks follow abnormal baseline elevations with insertion of a preceding wash cup (Brij/H_2O).

4) Annotate the chart record to identify the study, assay, specimens, data, instrument operation variables, and <u>date</u> of run.

5) Sign chart record which comprises the primary raw data of the assay.

6) Flush entire flow system using Brij/H_2O wash solution for a minimum of 30 min before shutdown.

7) Turn off power, release platen, and disengage pump tubes.

Data Processing

1) Measure peak heights on the chart record as the difference in the number of chart units (C.U.) between the baseline value immediately before each peak and the point of maximum pen deflection. (A transparent overlay transcribed with 0-100 divisions equivalent to those of the chart scale facilitates these measurements.)

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

2) Record C.U. values with corresponding identification numbers on Data Worksheets for subsequent calculation and evaluation procedures.

3) Record all other pertinent information and variables required for identification, calculations, results, and assay quality control.

4) Perform calculations as subsequently indicated in SOP and record results in format shown in Figures 2-5.

5) Assure compliance with requirements of Good Laboratory Practices (GLP) in maintenance and disposition of records and data.

Calculations

1) Calculate linear regression for GSH standard by the Method of Least Squares (9) expressed as ($y = mx + b$). Determine the correlation coefficient (r).

2) Use regression to calculate concentration values corresponding to peak heights of specimen and control cup preparations as follows:

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

$$C.U._{specimen} - C.U._{specimen} = \Delta\ C.U._{specimen}$$
$$+substrate \quad - substrate$$

$$\Delta\ C.U._{specimen} = (mx + b)\ \Delta\ umol\ SH/mL/T$$

T = Reaction time (min)

3) Calculate enzyme activities:

$$U\ ACHe/mL_{RBC} = \Delta\ umol\ SH/mL/min \times DF_{RBC}$$

$$U\ ACHe/mL_{plasma} = \Delta\ umol\ SH/mL/min \times DF_{plasma}$$

$$DF_{RBC} = \frac{Vol_{RBC}(mL) + Vol_{diluent}(mL)}{Vol_{RBC}(mL)}$$

$$DF_{plasma} = \frac{Vol_{plasma}(mL) + Vol_{diluent}(mL)}{Vol_{plasma}(mL)}$$

4) Use alternative formula to derive DF_{RBC} from HCT measurements when required

$$\text{Packed cell vol (PCV}^*) = Vol_{WB}(mL) \times HCT/_{100}$$

$$DF_{RBC} = \frac{(PCV + Vol^*_{diluent})}{PCV}$$

* in mL

5) Record results on data sheets in format shown in Figures 2-5.

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

Appendix A

Hardware components - AAII Chemistry Module - ACHe

1) 2 ea (#177B004-02) - 3 input connector

2) 2 ea (#177B0G4-01) - 2 input connector

3) 2 ea (#157B095-01) - 20 turn coil + terminal injection fitting

4) 2 ea (#A157-0202-01) - 1 turn phasing coil

5) 2 ea (#157-B273-01) - 37° C heating bath, B Coil (5.37 mL)

6) 2 ea (#157-B369-01/ #157B670-01) - 24" dialyzer assembly

7) 2 ea (#170-0103-01) - 5 turn mixing coil

8) 2 ea (#170-0472-02) - Type C dialyzer membranes

9) Misc glass tubing and plastic tubing for custom-fit connections

10) 2 ea AAII manifold trays, shell, covers, and heating bath mounting brackets.

List of Reference Procedure Modifications:

1) 50-fold dilution of Glutathione calibration standards.

2) Saline suspension of intact erythrocytes sampled into AAII system.

3) Separate colorimeters used to monitor absorbance activity in the presence and absence of substrate.

4) Two-channel recorder used for continuous, simultaneous chart record for both channels.

5) Shortened flow pathways prior to point of substrate addition.

6) 24 inch dialyzers

7) Glutathione Stds used to calibrate both channels.

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

(Fig 1)

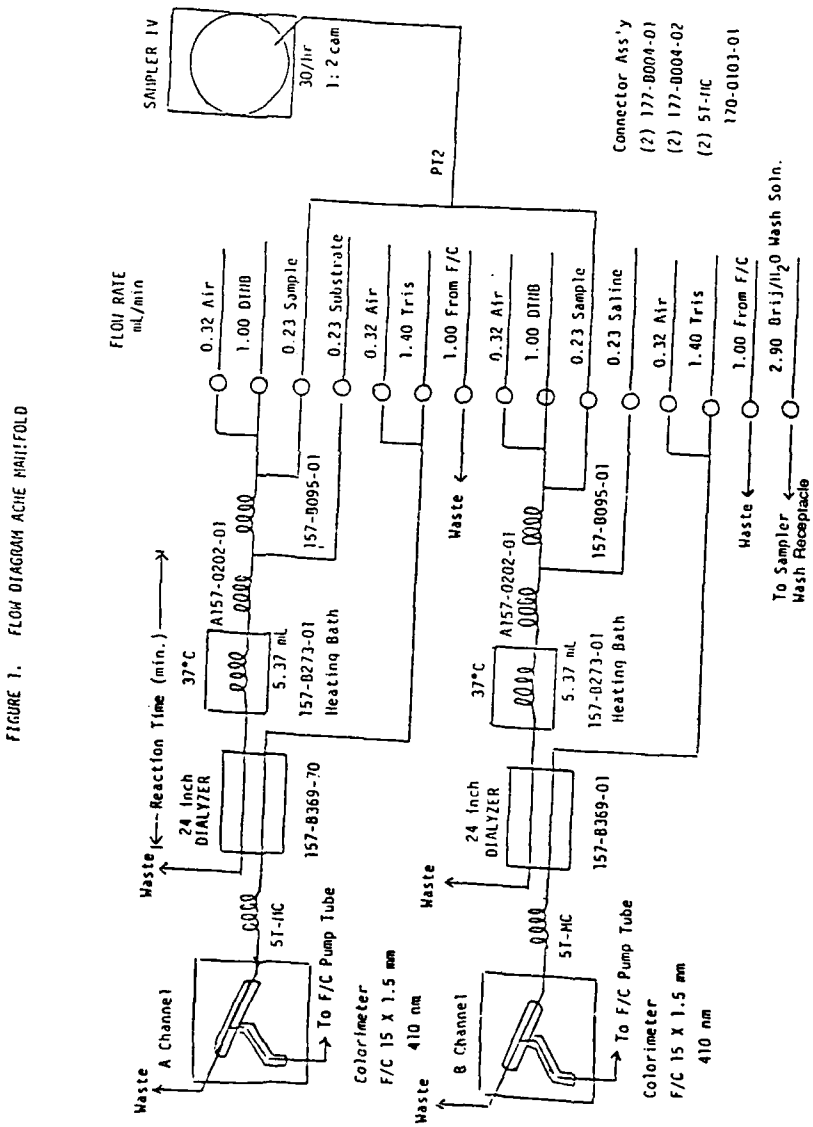


FIGURE 1. FLOW DIAGRAM ACHE MANIFOLD

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

(Fig 2)

PREPARATION OF SAMPLES

Study _____ Assay _____ Date _____ Analyst _____

Code _____ WB Vol. _____ uL ——→ PCV + _____ uL _____ DF= _____

Code _____ Vol. RBC _____ uL + _____ uL _____ DF= _____

Code _____ Vol. Plasma _____ uL + _____ uL _____ DF= _____

Code _____

Lab #	Specimen I.D. #	HCT R1	HCT R2	HCT Mean	RBC Prep Code	RBC DF	COMMENTS
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
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27							
28							
29							
30							

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

(Fig 3)

Study_____

Assay_____

Date_____

Analyst_____

STD_____ Stock Prep. Date_____ Stock Conc._____

STD Calibration

Lab I.D.#	X Values (umol/mL)	Y Values (Chart Units, C.U.) + Sustrate				- Substrate				

Linear Regression

(y = mx + b), y = _____ X + _____ Numbers of values, n = _____

Correlation Coefficient, r = _____

A II Reaction Time _____ min. at _____°C

Control _____ Stock Prep. Date _____ Stock Conc. _____

Lab I.D.#	Dilution	△ Chart Units					Activity (U/mL)

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

(Fig 4)

Chart Recorder Data

Page____of____Pages

Study_____

Date_____

Assay_____

Analyst_____

Lab #	CLP I.D.#	RBC +Subst.	RBC -Subst.	RBC △C.U.	PLASMA +Subst.	PLASMA -Subst.	PLASMA △C.U.	Notes
1								
2								
3								
4								
5								
6								
7								
8								
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Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

OP-ACH-83
Page 17 of 18
Feb 20, 1987

(Fig 5)

RESULTS

Study ______ SOP # ______

Assay ______ Species ______ Date ______

Specimen I.D. #	HCT	RBC UAChe/ml		Plasma UChe/mL	

Appendix G (cont.): PROCEDURES FOR CHOLINESTERASE DETERMINATIONS

SIGNATURES PAGE

Approved: 20 Feb 87
Date

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Chief, Quality Assurance Unit

Appendix H: PYRIDOSTIGMINE CONSUMPTION (mg/kg/day)

Animal# 86D00-	Group	WK1	WK2	WK3	WK4	WK5	WK6	WK7	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
517	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
532	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
538	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
540	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
558	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
576	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
578	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
579	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
584	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
585	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
614	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
647	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
665	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
715	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
725	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mean		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Std Dev		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SEM		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Appendix H (cont.): PYRIDOSTIGMINE CONSUMPTION (mg/kg/day)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23	WK24	WK25	WK26	WK27	WK28	WK29	WK30
517	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
532	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
538	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
540	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
558	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
576	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
578	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
579	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
584	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
585	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
614	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
647	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
665	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
715	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
725	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
Mean		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Std Dev		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SEM		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Appendix H (cont.): PYRIDOSTIGMINE CONSUMPTION (mg/kg/day)

Animal# 86D00-	Group	WK1	WK2	WK3	WK4	WK5	WK6	WK7	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
503	2	1.2	1.2	0.9	0.9	1.0	1.0	1.0	0.9	0.9	1.0	0.9	0.9	1.1	0.9	1.0
515	2	1.2	1.1	0.9	0.9	0.9	1.0	1.0	0.8	0.8	0.9	0.9	0.8	1.0	0.9	0.9
549	2	1.3	1.2	1.0	1.0	0.9	1.0	0.9	0.9	0.9	0.9	0.9	0.8	1.0	0.9	0.9
582	2	1.1	1.0	0.9	0.9	0.9	1.1	1.0	0.9	0.9	0.9	0.9	0.9	1.1	0.9	0.9
594	2	1.0	1.0	0.9	0.9	0.9	1.0	1.0	0.9	0.9	0.8	0.9	0.8	1.0	0.9	0.9
610	2	1.2	1.0	0.9	1.0	0.9	1.0	1.3	1.0	0.9	0.9	1.0	0.9	1.1	1.0	1.0
653	2	1.2	1.1	1.0	1.0	0.9	1.1	1.0	1.0	0.9	0.9	0.9	0.9	1.1	0.9	0.9
693	2	1.0	1.0	0.9	1.0	0.9	1.1	0.9	0.8	0.9	0.9	0.9	0.8	1.0	0.9	1.0
710	2	1.2	1.0	0.9	0.9	0.9	1.1	0.9	0.8	0.8	0.9	0.9	0.5	1.0	0.9	0.9
732	2	1.3	1.1	0.9	0.9	0.9	1.1	1.0	0.9	0.9	0.9	0.9	0.8	1.0	0.9	0.9
Mean		1.2	1.1	0.9	1.0	0.9	1.1	1.0	0.9	0.9	0.9	0.9	0.8	1.0	0.9	0.9
Std Dev		0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.1	0.1	0.0	0.0
SEM		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Appendix H (cont.): PYRIDOSTIGMINE CONSUMPTION (mg/kg/day)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23	WK24	WK25	WK26
503	2	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.1	1.0	•	1.3
515	2	0.9	0.9	0.9	0.9	0.9	1.0	0.9	0.9	0.9	1.0	1.0
549	2	1.0	0.9	1.0	1.0	1.0	1.1	0.9	1.0	1.1	0.6	1.3
582	2	1.0	0.9	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.1
594	2	0.9	0.9	0.9	0.8	0.9	1.0	0.9	0.9	1.0	1.1	1.1
610	2	1.0	1.0	1.1	1.1	1.1	1.2	1.1	1.2	1.2	1.3	1.4
653	2	0.9	0.9	0.9	0.8	1.0	1.0	1.0	1.1	1.1	1.1	1.2
693	2	1.0	0.9	0.9	0.9	0.9	1.0	0.9	1.0	•	1.0	1.1
710	2	0.9	0.8	0.9	1.0	0.9	1.0	0.9	1.0	1.0	1.1	1.1
732	2	0.9	0.9	0.9	1.0	1.0	1.1	0.8	1.0	1.0	1.1	1.0
Mean		1.0	0.9	1.0	1.0	1.0	1.1	0.9	1.0	1.0	1.0	1.2
Std Dev		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1
SEM		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0

• Unable to calculate due to incomplete food consumption data (spill).

Appendix H (cont.): PYRIDOSTIGMINE CONSUMPTION (mg/kg/day)

Animal# 86D00-	Group	WK1	WK2	WK3	WK4	WK5	WK6	WK7	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
534	3	10.9	10.6	9.9	11.2	9.8	10.6	11.2	11.5	10.5	10.7	10.0	9.8	11.3	10.0	10.1
536	3	8.8	9.2	9.2	10.0	8.5	10.4	10.8	10.6	8.9	10.3	10.2	9.9	11.4	9.7	10.1
559	3A	8.8	9.1	8.7	9.4	7.9	9.8	9.7	9.0	8.8	8.7	9.0	8.6	10.0	8.5	9.1
571	3A	7.8	7.5	8.3	8.1	7.8	9.1	9.9	8.9	8.6	8.9	8.6	8.9	9.2	8.8	9.4
572	3	8.9	7.9	8.4	9.1	8.0	10.0	10.5	10.4	9.6	9.5	10.0	9.4	10.5	9.2	9.8
603	3	10.5	10.3	9.3	9.6	8.7	10.7	10.4	10.4	9.5	9.2	9.6	10.0	10.4	8.8	9.3
608	3A	8.0	8.2	7.5	8.5	7.5	9.5	9.3	9.1	8.2	7.5	8.6	9.2	9.7	3.1	8.8
613	3	8.7	9.3	8.4	9.2	8.2	9.9	10.4	10.0	9.0	8.8	9.1	8.9	9.8	9.0	9.0
622	3A	9.7	8.6	6.8	9.0	8.3	10.7	10.4	10.2	9.9	9.4	9.7	9.5	10.4	9.1	9.9
638	3	11.0	10.2	9.2	9.9	9.0	10.7	10.9	9.9	9.4	8.3	8.6	9.6	9.8	9.6	10.1
651	3A	9.7	9.2	8.9	9.5	8.7	10.6	11.0	10.4	10.0	9.6	9.9	9.3	10.4	8.6	10.0
658	3	10.8	10.8	10.3	11.3	9.5	11.8	11.1	10.4	9.7	10.2	9.4	9.4	9.8	•	9.4
662	3	9.5	9.0	8.8	9.6	8.3	10.5	10.3	9.7	9.5	9.4	9.5	8.8	9.5	6.1	10.1
671	3	7.8	7.5	9.3	9.4	8.2	10.3	10.3	10.0	9.6	9.7	9.7	9.2	10.3	9.3	10.1
701	3	8.7	8.6	8.5	9.0	8.0	10.2	10.1	9.8	9.0	8.9	11.1	9.2	9.1	9.3	9.9
Mean		9.3	9.1	8.8	9.5	8.4	10.3	10.4	10.0	9.4	9.3	9.5	9.3	10.1	8.5	9.7
Std Dev		1.1	1.1	0.9	0.9	0.6	0.6	0.5	0.7	0.6	0.7	0.7	0.4	0.7	1.8	0.5
SEM		0.3	0.3	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.1	0.2	0.5	0.1

• Unable to calculate due to incomplete food consumption data (spill).

Appendix H (cont.): PYRIDOSTIGMINE CONSUMPTION (mg/kg/day)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23	WK24	WK25	WK26	WK27	WK28	WK29	WK30
534	3	9.9	11.4	10.8	10.8	11.6	9.9	10.3	9.3	•	10.8	9.7				
536	3	•	11.9	10.8	9.5	11.5	9.9	10.8	10.6	•	11.8	9.9				
559	3A	8.6	8.8	9.2	9.2	10.2	9.1	9.6	•	•	9.7	9.2	0.0	0.0	0.0	0.0
571	3A	8.8	10.2	9.9	9.1	9.0	9.3	9.5	8.7	•	10.2	10.0	0.0	0.0	0.0	0.0
572	3	9.9	9.9	9.8	9.3	10.5	•	10.1	8.8	9.2	10.8	9.8				
603	3	10.3	10.8	10.4	9.8	11.2	10.5	10.4	9.1	10.2	11.8	10.0				
608	3A	9.3	10.8	9.9	8.6	9.8	10.1	10.2	9.5	10.6	9.9	9.3	0.0	0.0	0.0	0.0
613	3	9.0	9.8	9.3	9.0	9.9	9.3	9.4	3.4	9.2	9.3	8.4				
622	3A	9.6	11.3	10.2	9.9	10.5	10.0	10.6	9.2	9.6	9.7	10.7	0.0	0.0	0.0	0.0
638	3	9.7	10.2	9.9	9.8	10.9	10.0	10.1	9.0	9.5	10.1	9.6				
651	3A	9.5	10.7	9.8	9.1	12.1	10.9	9.5	8.9	9.9	10.4	9.6	0.0	0.0	0.0	0.0
658	3	9.6	11.3	10.0	9.7	10.8	10.4	10.3	9.2	9.9	11.0	10.1				
662	3	9.8	9.4	9.2	7.6	9.3	8.5	9.1	8.4	7.8	9.3	9.1				
671	3	9.4	10.5	10.0	8.6	10.6	9.2	9.7	8.7	8.9	10.3	10.1				
701	3	9.3	10.3	9.5	9.3	9.9	9.4	10.2	8.4	9.0	10.0	9.4				
Mean		9.5	10.5	9.9	9.3	10.5	9.8	10.0	8.7	9.4	10.3	9.7	0.0	0.0	0.0	0.0
Std Dev		0.5	0.8	0.5	0.7	0.9	0.7	0.5	1.6	0.8	0.8	0.6	0.0	0.0	0.0	0.0
SEM		0.1	0.2	0.1	0.2	0.2	0.2	0.1	0.4	0.2	0.2	0.1	0.0	0.0	0.0	0.0

• Unable to calculate due to incomplete food consumption data (spill).

Appendix H (cont.): PYRIDOSTIGMINE CONSUMPTION (mg/kg/day)

Animal# 86D00-	Group	WK1	WK2	WK3	WK4	WK5	WK6	WK7	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
516	7	8.4	9.0	8.1	8.5	7.3	9.8	10.3	9.3	9.0	9.3	9.1	9.3	11.4	9.5	8.8
518	7A	9.3	8.4	6.9	8.0	8.7	9.2	11.0	9.6	8.7	9.2	8.9	8.8	10.3	9.3	8.9
524	7A	9.2	8.9	7.2	8.3	8.6	9.5	11.2	9.9	9.3	9.6	9.2	8.9	10.8	10.0	9.3
535	7	9.6	8.5	7.3	9.0	9.6	10.4	11.3	9.9	9.6	9.4	9.3	9.8	11.8	10.4	10.4
537	7A	10.3	9.7	6.9	8.0	9.4	10.3	11.0	9.7	8.9	9.2	8.3	9.7	10.4	9.2	9.2
547	7A	8.8	9.0	6.9	8.6	9.5	10.2	11.1	10.1	9.6	9.6	9.7	9.6	11.1	9.7	9.0
560	7	9.3	8.5	6.8	8.7	9.4	9.7	10.9	9.5	9.0	9.1	9.2	9.5	11.1	10.0	9.7
569	7	9.9	8.6	6.7	8.6	8.6	10.2	11.1	9.4	9.2	8.6	8.8	8.7	9.8	9.4	9.3
591	7A	12.0	9.2	7.1	9.3	9.2	9.9	11.1	10.1	9.2	8.8	9.7	9.9	11.5	10.2	9.6
597	7	11.9	9.8	8.6	8.7	9.0	11.4	11.4	10.0	10.9	10.1	9.6	10.4	12.2	10.6	9.7
616	7	9.2	7.4	7.5	7.5	8.5	8.8	9.7	8.2	8.2	8.1	8.8	9.1	10.1	10.2	8.1
623	7	8.8	7.6	7.7	8.1	8.3	9.0	9.9	9.4	7.9	9.1	9.3	6.6	10.9	10.8	8.5
654	7	9.8	8.0	8.2	8.8	8.7	9.6	10.4	9.0	8.9	8.2	8.2	8.6	9.4	9.7	8.1
660	7	10.6	8.6	8.4	8.3	8.5	9.6	10.5	9.2	9.0	9.0	9.0	9.2	10.7	10.6	9.4
729	7	9.2	8.3	7.2	7.8	9.1	8.8	9.9	8.8	8.1	8.4	6.5	9.8	10.7	10.9	9.1
Mean		9.8	8.6	7.4	8.4	8.8	9.8	10.7	9.5	9.0	9.1	8.9	9.2	10.8	10.0	9.1
Std Dev		1.1	0.7	0.6	0.5	0.6	0.7	0.6	0.5	0.7	0.5	0.8	0.9	0.8	0.6	0.6
SEM		0.3	0.2	0.2	0.1	0.2	0.2	0.2	0.1	0.2	0.1	0.2	0.2	0.2	0.1	0.2

Appendix H (cont.): PYRIDOSTIGMINE CONSUMPTION (mg/kg/day)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23	WK24	WK25	WK26	WK27	WK28	WK29	WK30
516	7	10.7	10.1	11.3	11.1	9.4	11.3	10.6	10.7	10.8	10.3	9.7				
518	7A	11.4	10.3	11.5	10.4	10.1	10.3	10.6	10.2	9.8	10.0	9.8	0.0	0.0	0.0	0.0
524	7A	10.8	9.4	10.0	10.3	11.0	10.6	10.0	10.0	11.9	10.2	8.9	0.0	0.0	0.0	0.0
535	7	11.0	10.8	12.6	10.5	10.9	11.0	11.2	11.1	11.4	11.0	9.9				
537	7A	9.8	9.0	8.8	9.5	10.4	10.6	10.0	11.0	10.9	10.2	10.1	0.0	0.0	0.0	0.0
547	7A	10.7	11.0	11.0	10.2	10.1	10.6	10.7	6.8	11.4	11.2	10.1	0.0	0.0	0.0	0.0
560	7	10.5	10.3	11.2	10.0	10.4	10.1	10.4	10.7	10.4	11.2	9.3				
569	7	9.3	10.3	10.5	10.7	10.5	11.6	11.1	10.7	10.3	10.4	8.6				
591	7A	10.1	10.5	10.3	9.6	10.4	9.7	10.7	10.6	10.5	10.4	9.5	0.0	0.0	0.0	0.0
597	7	9.7	11.4	11.3	11.7	10.3	9.6	11.0	10.9	•	9.1	9.0				
616	7	9.3	8.4	9.7	9.9	9.1	7.7	9.1	9.7	9.5	10.3	7.8				
623	7	9.2	8.9	10.5	9.4	9.2	10.1	9.7	9.9	9.7	9.9	8.8				
654	7	9.7	8.3	9.5	9.9	8.6	9.2	10.1	9.3	10.5	8.1	8.1				
660	7	9.4	9.9	10.8	9.7	10.0	10.5	11.3	10.7	11.1	9.9	8.9				
729	7	10.2	10.1	10.2	9.5	9.5	9.7	9.5	10.3	10.9	9.3	9.1				
Mean		10.1	9.9	10.6	10.2	10.0	10.2	10.4	10.2	10.7	10.1	9.2	0.0	0.0	0.0	0.0
Std Dev		0.7	0.9	0.9	0.7	0.7	0.9	0.7	1.1	0.7	0.8	0.7	0.0	0.0	0.0	0.0
SEM		0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.0	0.0	0.0	0.0

• Unable to calculate due to incomplete food consumption data (spill).

Appendix I: FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	QWK2[@]	WK1	WK2	WK3	WK4	WK5	WK6	WK7
517	1	146	157	166	168	179	171	177	190
532	1	146	162	164	173	173	168	171	185
538	1	156	166	173	180	180	189	184	185
540	1	132	162	158	156	165	162	147	159
558	1	137	152	154	161	165	164	spill	168
576	1	168	158	175	182	189	190	198	197
578	1	139	175	218	178	172	177	175	183
579	1	163	161	192	192	193	189	186	188
584	1	159	145	157	164	180	189	178	190
585	1	146	148	164	159	148	145	137	141
614	1	37	183	189	193	202	204	205	210
647	1	139	144	167	160	179	174	173	173
665	1	148	117	168	164	161	181	180	168
715	1	144	155	151	150	154	162	157	150
725	1	142	171	184	176	175	181	168	179
Mean		140.1	157.1	172.0	170.4	174.3	176.4	174.0	177.7
Std Dev		30.2	15.5	17.6	12.9	14.4	14.9	18.1	18.2
SEM		7.8	4.0	4.6	3.3	3.7	3.9	4.8	4.7

[@] Quarantine week 2.

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
517	1	178	178	178	175	177	181	183	174
532	1	186	188	198	spill	197	183	243	123
538	1	188	186	167	95	186	184	176	177
540	1	164	168	171	161	163	165	165	168
558	1	176	167	168	167	166	167	167	167
576	1	197	200	210	196	194	158	183	210
578	1	176	170	177	167	172	171	165	171
579	1	179	177	181	spill	178	248	167	176
584	1	193	189	189	193	200	204	190	189
585	1	145	142	136	133	132	137	133	139
614	1	221	209	213	203	218	223	214	216
647	1	165	167	179	169	170	159	170	167
665	1	176	167	138	171	162	176	168	180
715	1	155	70	158	156	162	161	162	156
725	1	169	201	214	151	359	171	171	179
Mean		177.9	171.9	178.5	164.4	189.1	179.2	177.1	172.8
Std Dev		18.3	32.9	24.1	28.2	51.3	27.7	25.0	23.3
SEM		4.7	8.5	6.2	7.8	13.2	7.2	6.5	6.0

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23
517	1	175	173	176	160	174	177	178	175
532	1	184	189	188	177	244	204	185	180
538	1	180	169	186	176	184	186	180	183
540	1	164	149	155	143	158	161	150	160
558	1	159	160	156	155	201	159	158	162
576	1	204	203	191	183	198	211	204	204
578	1	180	169	164	162	174	175	173	175
579	1	191	160	163	165	177	181	173	193
584	1	184	180	187	175	185	202	190	193
585	1	130	134	138	141	144	144	147	140
614	1	210	205	191	189	205	210	187	213
647	1	151	156	159	154	156	161	162	161
665	1	164	146	172	179	182	181	174	184
715	1	158	145	161	163	160	221	159	165
725	1	162	157	168	177	178	187	167	168
Mean		173.1	166.3	170.3	166.6	181.3	184.0	172.5	177.1
Std Dev		20.8	20.9	15.9	14.4	24.4	22.3	15.7	18.9
SEM		5.4	5.4	4.1	3.7	6.3	5.7	4.0	4.9

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK24	WK25	WK26	WK27	WK28	WK29	WK30
517	1	169	175	181				
532	1	196	168	189				
538	1	148	184	181				
540	1	147	167	178	147	137	131	133
558	1	148	164	157				
576	1	206	207	214				
578	1	170	180	177				
579	1	186	182	175				
584	1	186	197	193				
585	1	151	145	150	457	111	123	123
614	1	209	217	216	205	194	185	191
647	1	179	152	167	161	137	136	130
665	1	189	175	188	160	153	154	150
715	1	170	170	174				
725	1	179	158	166				
Mean		175.5	176.1	180.4	226.0	146.4	145.8	145.4
Std Dev		20.6	19.6	18.2	131.0	30.6	24.7	27.4
SEM		5.3	5.1	4.7	58.6	13.7	11.0	12.2

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	QWK2[@]	WK1	WK2	WK3	WK4	WK5	WK6	WK7
503	2	135	153	175	174	174	184	163	167
515	2	161	200	201	203	212	205	207	214
549	2	102	140	157	161	165	161	152	157
582	2	155	168	177	176	180	174	181	177
594	2	159	175	191	195	205	191	194	199
610	2	127	140	143	142	148	143	142	200
653	2	127	151	167	174	185	176	182	187
693	2	152	153	161	167	176	168	172	171
710	2	170	211	188	197	197	200	201	195
732	2	123	144	157	159	161	166	175	179
Mean		141.1	163.5	171.7	174.8	180.3	176.8	176.9	184.6
Std Dev		21.5	24.9	18.0	19.1	20.1	18.8	20.8	17.5
SEM		6.8	7.9	5.7	6.0	6.3	5.9	6.6	5.5

[@] Quarantine week 2.

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
503	2	173	177	181	170	164	182	174	175
515	2	205	208	225	218	202	211	207	207
549	2	175	174	170	161	146	154	161	158
582	2	185	177	181	173	180	179	177	171
594	2	200	203	192	194	194	198	187	198
610	2	163	160	151	158	150	157	152	148
653	2	199	190	184	178	179	182	179	165
693	2	170	175	180	169	165	167	182	179
710	2	195	190	207	203	115	193	191	186
732	2	181	185	187	187	162	180	185	172
Mean		184.6	183.9	185.8	181.1	165.7	180.3	179.5	175.9
Std Dev		14.5	14.4	19.9	19.2	25.2	17.8	15.3	17.7
SEM		4.6	4.6	6.3	6.1	8.0	5.6	4.8	5.6

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23
503	2	176	178	175	167	171	192	179	180
515	2	207	216	214	201	213	239	204	204
549	2	161	164	170	158	165	180	163	167
582	2	179	170	177	175	183	188	174	172
594	2	192	193	201	182	209	208	204	194
610	2	153	159	161	161	158	168	163	164
653	2	167	181	172	151	178	187	181	189
693	2	184	177	166	175	181	190	177	178
710	2	196	177	188	197	193	198	181	197
732	2	169	185	181	182	191	211	166	184
Mean		178.4	180.0	180.5	174.9	184.2	196.1	179.2	182.9
Std Dev		16.7	16.0	16.4	16.3	17.8	19.6	14.8	13.2
SEM		5.3	5.1	5.2	5.1	5.6	6.2	4.7	4.2

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK24	WK25	WK26	WK27	WK28	WK29	WK30
503	2	187	spill	190				
515	2	209	197	200				
549	2	174	89	171				
582	2	173	168	169				
594	2	207	213	200				
610	2	173	165	169				
653	2	194	190	191				
693	2	spill	176	172				
710	2	204	195	192				
732	2	188	199	169				
Mean		189.9	176.9	182.3				
Std Dev		14.6	36.5	13.4				
SEM		4.9	12.2	4.2				

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	QWK2[@]	WK1	WK2	WK3	WK4	WK5	WK6	WK7
534	3	129	155	179	182	205	200	181	183
536	3	144	157	171	176	186	170	171	172
559	3A	140	163	180	181	191	172	176	168
571	3A	157	165	154	176	173	177	171	181
572	3	135	142	133	145	152	143	151	156
603	3	117	134	155	154	162	164	170	165
608	3A	140	151	154	142	155	144	149	140
613	3	140	152	170	158	170	165	168	172
622	3A	154	191	178	145	184	182	194	182
638	3	134	158	168	166	179	178	178	176
651	3A	148	175	174	174	184	184	187	189
658	3	143	153	170	174	192	178	187	173
662	3	132	174	177	181	195	185	197	188
671	3	144	141	138	178	177	167	174	170
701	3	155	166	172	178	185	175	188	181
Mean		140.8	158.5	164.9	167.3	179.3	172.3	176.1	173.1
Std Dev		10.6	14.7	14.8	14.5	14.9	14.8	13.9	12.8
SEM		2.7	3.8	3.8	3.8	3.8	3.8	3.6	3.3

[@] Quarantine week 2.

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
534	3	208	203	210	190	183	201	194	188
536	3	184	159	186	177	170	183	170	170
559	3A	167	171	170	168	160	174	158	164
571	3A	176	175	183	167	173	171	175	178
572	3	167	159	161	166	155	163	156	159
603	3	182	175	176	176	183	181	167	166
608	3A	146	135	124	134	142	142	48	127
613	3	181	168	167	166	162	169	168	162
622	3A	192	197	191	188	181	188	178	185
638	3	175	173	172	171	172	165	174	178
651	3A	195	197	193	187	173	184	165	184
658	3	175	173	185	162	157	154	spill	153
662	3	194	200	202	196	180	183	124	195
671	3	181	182	187	180	171	182	180	188
701	3	190	182	180	207	170	165	180	185
Mean		180.9	176.6	179.1	175.7	168.8	173.7	159.8	172.1
Std Dev		14.7	18.2	20.0	17.3	11.7	14.7	35.9	17.6
SEM		3.8	4.7	5.2	4.5	3.0	3.8	9.6	4.6

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23
534	3	187	195	195	207	209	187	190	193
536	3	spill	180	174	160	181	166	175	195
559	3A	157	142	157	166	173	162	165	spill
571	3A	169	176	182	177	163	176	174	180
572	3	164	147	153	153	163	spill	159	155
603	3	188	179	181	179	192	191	183	178
608	3A	136	145	142	127	132	143	140	148
613	3	163	158	160	162	165	165	161	66
622	3A	182	194	188	190	189	190	196	192
638	3	174	165	169	176	185	180	177	179
651	3A	176	178	173	167	211	202	170	178
658	3	158	168	159	162	169	173	164	165
662	3	199	172	179	155	176	170	178	186
671	3	179	180	183	165	191	176	180	182
701	3	179	180	176	181	181	180	189	177
Mean		172.2	170.6	171.4	168.5	178.7	175.8	173.4	169.6
Std Dev		15.9	16.5	14.5	18.2	19.7	14.7	14.3	32.8
SEM		4.3	4.2	3.8	4.7	5.1	3.9	3.7	8.8

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK24	WK25	WK26	WK27	WK28	WK29	WK30
534	3	spill	195	179				
536	3	spill	191	164				
559	3A	spill	168	163	147	146	140	132
571	3A	spill	189	190	160	150	153	144
572	3	151	169	155				
603	3	185	206	178				
608	3A	156	138	131	123	127	113	124
613	3	168	161	147				
622	3A	187	177	201	168	165	151	149
638	3	177	177	173				
651	3A	185	185	176	159	140	140	134
658	3	166	174	165				
662	3	162	183	184				
671	3	174	191	194				
701	3	175	187	180				
Mean		171.5	179.4	172.0	151.4	145.6	139.4	136.6
Std Dev		12.0	16.4	18.4	17.6	13.9	15.9	9.9
SEM		3.6	4.2	4.8	7.9	6.2	7.1	4.4

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	QWK2[@]	WK1	WK2	WK3	WK4	WK5	WK6	WK7
516	7	165	157	188	172	174	130	172	169
518	7A	160	174	186	161	186	179	182	203
524	7A	134	153	169	141	162	151	161	176
535	7	157	185	186	162	196	182	189	192
537	7A	131	151	175	133	153	157	168	168
547	7A	150	153	179	143	176	172	179	186
560	7	158	175	185	153	196	189	193	206
569	7	134	155	155	123	155	139	160	164
591	7A	159	197	190	157	207	185	195	206
597	7	95	137	150	148	154	146	188	174
616	7	155	179	168	176	176	175	175	182
623	7	146	167	164	171	178	161	172	179
654	7	141	155	153	167	181	162	176	181
660	7	135	155	154	162	160	148	164	169
729	7	139	158	167	151	162	168	157	164
Mean		143.9	163.4	171.3	154.7	174.4	162.9	175.4	181.3
Std Dev		17.6	15.7	14.0	15.1	16.8	17.7	12.2	14.6
SEM		4.5	4.1	3.6	3.9	4.3	4.6	3.2	3.8

[@] Quarantine week 2.

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
516	7	172	164	172	167	160	174	158	158
518	7A	203	185	197	188	174	181	181	189
524	7A	181	171	181	174	158	169	172	174
535	7	195	190	189	186	183	197	192	211
537	7A	171	157	164	147	162	154	150	165
547	7A	195	183	183	183	168	175	169	169
560	7	209	195	200	203	199	209	207	221
569	7	161	158	151	153	143	141	147	157
591	7A	214	195	190	209	202	209	206	211
597	7	168	181	178	181	181	189	188	179
616	7	175	172	174	186	183	181	202	175
623	7	195	161	190	193	129	189	211	183
654	7	178	178	167	162	162	160	181	162
660	7	172	171	175	175	168	174	190	185
729	7	167	155	164	126	175	172	195	178
Mean		183.7	174.4	178.3	175.5	169.8	178.3	183.3	181.1
Std Dev		16.7	13.6	13.6	21.8	19.2	18.8	20.5	19.7
SEM		4.3	3.5	3.5	5.6	5.0	4.9	5.3	5.1

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23
516	7	160	148	167	172	147	167	157	162
518	7A	206	182	202	192	193	186	190	188
524	7A	169	143	151	165	182	164	154	158
535	7	190	182	211	185	197	186	189	193
537	7A	148	132	129	146	164	157	147	167
547	7A	169	169	168	164	164	161	160	104
560	7	203	195	213	199	211	192	196	209
569	7	133	144	147	157	157	162	155	153
591	7A	188	193	189	183	204	178	195	199
597	7	146	168	169	186	168	146	167	174
616	7	169	151	174	185	175	140	165	181
623	7	167	158	185	172	172	176	167	175
654	7	164	137	157	171	151	153	167	158
660	7	157	162	176	165	178	175	186	182
729	7	171	167	169	164	168	160	158	176
Mean		169.3	162.1	173.8	173.7	175.4	166.9	170.2	171.9
Std Dev		20.4	19.8	23.5	14.3	19.0	15.1	16.5	24.7
SEM		5.3	5.1	6.1	3.7	4.9	3.9	4.2	6.4

Appendix I (cont.): FOOD CONSUMPTION (g/week)

Animal# 86D00-	Group	WK24	WK25	WK26	WK27	WK28	WK29	WK30
516	7	164	160	160				
518	7A	176	181	192	168	169	161	140
524	7A	188	165	153	152	134	123	123
535	7	199	197	188				
537	7A	167	160	169	136	55	132	137
547	7A	174	176	168	145	140	143	139
560	7	203	225	199				
569	7	147	151	132				
591	7A	199	202	196	179	186	173	146
597	7	spill	146	153				
616	7	176	195	158				
623	7	169	176	169				
654	7	179	141	150				
660	7	188	172	164				
729	7	185	161	168				
Mean		179.6	173.9	167.9	156.0	136.8	146.4	137.0
Std Dev		15.5	23.1	18.8	17.4	50.4	20.5	8.5
SEM		4.1	6.0	4.9	7.8	22.5	9.2	3.8

Appendix J: WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	QWK2[@]	WK1	WK2	WK3	WK4	WK5	WK6	WK7
517	1	174	191	195	207	215	186	192	189
532	1	220	250	244	265	291	303	324	341
538	1	202	225	251	261	254	254	257	238
540	1	200	286	283	278	290	293	270	268
558	1	166	197	200	223	228	243	256	238
576	1	246	259	257	324	323	323	325	325
578	1	182	208	311	230	222	235	210	212
579	1	229	251	262	281	277	252	264	261
584	1	271	312	296	307	339	359	348	336
585	1	291	252	248	247	244	207	192	206
614	1	225	250	257	272	291	284	280	295
647	1	206	236	245	248	272	254	235	224
665	1	236	254	247	275	282	264	264	276
715	1	184	190	201	211	217	219	216	193
725	1	186	217	225	236	241	238	208	208
Mean		214.5	238.5	248.1	257.7	265.7	260.9	256.1	254.0
Std Dev		35.9	34.7	33.5	33.5	38.2	45.3	48.8	51.6
SEM		9.3	9.0	8.7	8.7	9.9	11.7	12.6	13.3

[@] Quarantine week 2.

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
517	1	193	171	178	178	178	183	182	170
532	1	337	327	335	331	327	293	346	262
538	1	237	224	201	218	231	208	214	206
540	1	257	258	242	245	236	235	236	241
558	1	244	225	232	219	253	227	258	208
576	1	323	265	305	301	310	200	263	255
578	1	229	208	203	216	196	190	188	194
579	1	242	256	247	191	231	222	227	229
584	1	321	344	354	357	353	305	301	327
585	1	261	212	188	179	180	170	166	182
614	1	294	287	281	297	304	325	294	302
647	1	229	213	218	204	215	189	193	206
665	1	279	250	247	247	251	249	291	246
715	1	212	196	210	185	211	213	196	178
725	1	191	211	232	195	202	194	178	195
Mean		256.6	243.1	244.9	237.5	245.2	226.9	235.5	226.7
Std Dev		45.9	48.0	52.5	57.8	54.6	47.0	54.4	45.7
SEM		11.9	12.4	13.6	14.9	14.1	12.1	14.0	11.8

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23
517	1	168	165	162	157	182	175	168	174
532	1	278	303	301	275	331	289	282	282
538	1	198	185	196	187	224	226	206	202
540	1	213	211	214	196	239	216	193	209
558	1	191	192	183	179	228	209	203	186
576	1	263	299	239	245	293	328	303	273
578	1	192	189	182	178	196	203	185	210
579	1	237	213	234	227	270	327	233	265
584	1	289	289	302	311	349	318	308	337
585	1	124	168	183	181	191	191	172	184
614	1	260	292	288	284	323	339	290	345
647	1	214	190	185	186	202	188	188	201
665	1	238	240	250	238	288	251	278	278
715	1	186	204	203	225	236	218	237	248
725	1	170	173	183	200	212	200	193	208
Mean		214.7	220.9	220.3	217.9	250.9	245.2	229.3	240.1
Std Dev		45.9	50.5	46.6	45.1	54.5	58.5	50.1	54.6
SEM		11.9	13.0	12.0	11.7	14.1	15.1	12.9	14.1

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK24	WK25	WK26	WK27	WK28	WK29	WK30
517	1	158	162	156				
532	1	306	273	277				
538	1	231	227	206				
540	1	201	228	203	191	225	180	199
558	1	208	197	185				
576	1	335	323	288				
578	1	200	201	190				
579	1	257	244	218				
584	1	321	323	269				
585	1	203	192	177	464	128	149	159
614	1	321	313	321	148	336	288	292
647	1	203	189	178	200	154	187	183
665	1	293	274	276	spill	282	306	251
715	1	245	247	209				
725	1	202	190	165				
Mean		245.6	238.9	221.2	250.8	225.0	222.0	216.8
Std Dev		56.2	52.5	51.4	144.0	86.6	70.2	53.9
SEM		14.5	13.6	13.3	72.0	38.7	31.4	24.1

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	QWK2[@]	WK1	WK2	WK3	WK4	WK5	WK6	WK7
503	2	312	354	382	304	476	297	308	312
515	2	spill	277	292	294	309	302	308	327
549	2	190	218	228	232	229	211	206	216
582	2	217	232	231	244	239	233	235	229
594	2	244	289	313	348	353	345	340	380
610	2	183	214	215	233	222	227	231	349
653	2	185	217	225	237	240	214	218	205
693	2	190	202	223	245	246	239	229	227
710	2	235	306	243	301	302	278	280	286
732	2	198	270	283	279	284	280	285	302
Mean		217.1	257.9	263.5	271.7	290.0	262.6	264.0	283.3
Std Dev		41.9	49.5	53.6	39.5	77.8	44.5	45.9	61.0
SEM		14.0	15.6	17.0	12.5	24.6	14.1	14.5	19.3

[@] Quarantine week 2.

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
503	2	272	254	250	294	267	302	259	249
515	2	288	290	285	297	301	277	280	298
549	2	216	208	208	204	196	204	204	192
582	2	232	212	221	221	232	206	205	326
594	2	363	341	368	369	337	331	281	328
610	2	247	235	225	241	242	228	224	203
653	2	222	219	207	199	204	195	182	180
693	2	223	230	246	212	213	215	215	221
710	2	289	272	268	288	296	286	281	265
732	2	315	285	261	260	268	279	274	251
Mean		266.7	254.6	253.9	258.5	255.6	252.3	240.5	251.3
Std Dev		47.9	42.4	47.8	54.0	46.4	48.1	38.4	53.4
SEM		15.1	13.4	15.1	17.1	14.7	15.2	12.1	16.9

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23
503	2	221	240	271	246	248	253	252	262
515	2	288	285	303	282	301	311	279	307
549	2	200	212	236	214	222	223	213	210
582	2	216	211	226	232	256	227	220	216
594	2	290	314	363	361	330	325	311	318
610	2	213	237	255	236	253	247	219	210
653	2	173	186	208	169	207	192	211	199
693	2	225	221	212	223	236	211	220	219
710	2	263	258	297	284	306	296	256	267
732	2	272	289	289	288	314	360	282	290
Mean		236.1	245.3	266.0	253.5	267.3	264.5	246.3	249.8
Std Dev		39.7	40.6	48.5	52.6	42.3	55.4	35.2	44.5
SEM		12.6	12.8	15.3	16.6	13.4	17.5	11.1	14.1

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK24	WK25	WK26	WK27	WK28	WK29	WK30
503	2	277	275	249				
515	2	310	289	280				
549	2	226	spill	244				
582	2	231	217	214				
594	2	335	361	321				
610	2	242	250	213				
653	2	219	202	185				
693	2	219	215	205				
710	2	275	291	237				
732	2	268	300	247				
Mean		260.2	266.7	239.5				
Std Dev		40.0	50.9	39.4				
SEM		12.6	17.0	12.5				

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	QWK2[@]	WK1	WK2	WK3	WK4	WK5	WK6	WK7
534	3	211	260	310	316	354	357	351	380
536	3	202	240	263	270	279	269	274	267
559	3A	219	261	282	298	294	259	279	262
571	3A	237	265	268	278	310	260	278	265
572	3	253	271	280	311	292	279	315	306
603	3	257	325	314	289	312	284	306	279
608	3A	187	189	183	185	181	155	163	162
613	3	183	206	215	207	221	207	198	202
622	3A	200	252	278	240	258	270	276	263
638	3	221	242	249	234	233	222	255	218
651	3A	201	254	273	269	282	256	266	260
658	3	219	239	271	281	265	235	239	211
662	3	222	249	255	247	238	214	227	224
671	3	184	224	spill	287	273	232	258	266
701	3	194	228	230	239	240	215	234	229
Mean		212.7	247.0	262.2	263.4	268.8	247.6	261.3	252.9
Std Dev		23.2	31.1	35.0	37.5	42.7	45.6	46.3	50.5
SEM		6.0	8.0	9.4	9.7	11.0	11.8	12.0	13.0

[@] Quarantine week 2.

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
534	3	356	325	304	317	317	312	297	278
536	3	267	239	270	253	269	247	227	220
559	3A	244	231	256	262	268	239	219	275
571	3A	221	222	233	239	265	194	219	215
572	3	309	304	308	315	282	269	263	239
603	3	292	341	303	287	303	265	234	235
608	3A	151	152	151	154	155	141	126	120
613	3	209	182	188	192	185	191	179	179
622	3A	272	322	285	282	269	308	264	269
638	3	215	191	188	199	184	186	175	187
651	3A	255	267	248	249	225	226	199	219
658	3	225	206	203	220	178	204	210	190
662	3	217	222	228	210	203	213	162	228
671	3	269	265	253	248	263	249	239	244
701	3	234	226	217	276	232	199	224	227
Mean		249.1	246.3	242.3	246.9	239.9	229.5	215.8	221.7
Std Dev		48.7	56.4	47.4	46.0	49.4	47.0	43.5	41.2
SEM		12.6	14.6	12.2	11.9	12.8	12.1	11.2	10.6

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23
534	3	269	321	304	328	309	309	292	281
536	3	215	217	227	219	239	235	238	249
559	3A	233	220	239	248	245	252	243	256
571	3A	202	218	217	200	189	238	200	228
572	3	252	245	260	284	248	287	276	273
603	3	286	256	257	256	275	300	272	274
608	3A	147	153	145	135	143	155	138	151
613	3	172	177	183	190	196	201	179	188
622	3A	242	303	312	308	301	316	302	303
638	3	175	167	175	184	180	185	232	178
651	3A	205	219	207	244	259	254	214	221
658	3	179	253	218	222	213	233	190	200
662	3	spill	198	202	190	200	208	214	222
671	3	229	230	244	248	276	257	250	259
701	3	231	227	240	250	234	238	246	231
Mean		216.9	226.9	228.7	233.7	233.8	244.5	232.4	234.3
Std Dev		39.6	45.6	44.9	50.5	46.9	45.9	44.6	42.3
SEM		10.6	11.8	11.6	13.0	12.1	11.9	11.5	10.9

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK24	WK25	WK26	WK27	WK28	WK29	WK30
534	3	304	289	289				
536	3	233	222	226				
559	3A	234	253	271	216	225	246	211
571	3A	226	243	226	182	160	219	207
572	3	269	246	236				
603	3	270	302	265				
608	3A	166	149	133	131	122	130	141
613	3	181	183	146				
622	3A	306	311	311	253	213	240	285
638	3	177	178	184				
651	3A	232	222	215	189	171	189	189
658	3	196	236	195				
662	3	211	208	215				
671	3	255	260	259				
701	3	237	235	230				
Mean		233.1	235.8	226.7	194.2	178.2	204.8	206.6
Std Dev		42.9	45.3	49.2	45.0	41.7	47.4	51.9
SEM		11.1	11.7	12.7	20.1	18.6	21.2	23.2

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	QWK2@	WK1	WK2	WK3	WK4	WK5	WK6	WK7
516	7	229	247	326	276	263	238	254	244
518	7A	240	278	257	269	276	276	296	295
524	7A	179	176	273	269	264	262	264	279
535	7	235	276	270	274	276	266	265	254
537	7A	284	309	318	287	271	250	264	260
547	7A	337	368	406	435	424	264	442	416
560	7	198	238	238	244	242	241	277	270
569	7	176	202	206	194	193	190	203	198
591	7A	275	378	393	382	372	353	355	354
597	7	180	261	323	380	443	253	384	446
616	7	191	219	214	224	220	199	205	201
623	7	180	222	209	220	228	220	237	240
654	7	184	237	247	236	248	231	231	235
660	7	155	185	241	206	210	192	194	203
729	7	202	239	243	248	270	259	241	257
Mean		216.3	255.7	277.6	276.3	280.0	246.3	274.1	276.8
Std Dev		50.2	59.2	62.8	69.9	74.5	40.4	70.0	74.2
SEM		13.0	15.3	16.2	18.1	19.2	10.4	18.1	19.2

@ Quarantine week 2.

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK8	WK9	WK10	WK11	WK12	WK13	WK14	WK15
516	7	spill	235	238	254	190	211	221	193
518	7A	283	258	260	260	266	263	263	258
524	7A	292	256	296	273	255	251	261	272
535	7	285	248	245	277	261	230	252	237
537	7A	258	232	240	243	253	220	232	223
547	7A	417	422	447	441	428	395	399	368
560	7	242	221	266	263	257	245	251	253
569	7	194	173	191	202	207	169	189	177
591	7A	360	330	330	365	349	331	333	316
597	7	441	437	spill	spill	462	spill	spill	416
616	7	195	192	196	205	204	191	184	181
623	7	234	203	225	226	168	234	230	209
654	7	235	222	210	211	210	213	203	203
660	7	207	199	204	192	185	194	192	194
729	7	272	285	296	spill	358	306	301	282
Mean		279.6	260.9	260.3	262.5	270.2	246.6	250.8	252.1
Std Dev		77.1	78.7	67.6	70.2	89.7	61.0	60.3	70.0
SEM		20.6	20.3	18.1	19.5	23.2	16.3	16.1	18.1

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK16	WK17	WK18	WK19	WK20	WK21	WK22	WK23
516	7	199	188	223	239	190	220	216	223
518	7A	246	239	244	240	230	224	228	235
524	7A	269	260	285	301	277	280	259	267
535	7	213	237	222	212	229	221	227	211
537	7A	234	219	223	235	232	234	233	246
547	7A	357	408	376	391	378	404	475	347
560	7	236	242	242	236	245	240	234	229
569	7	171	178	175	195	186	166	186	169
591	7A	301	334	296	298	325	310	309	312
597	7	431	214	spill	414	471	417	196	424
616	7	174	204	196	210	214	189	211	199
623	7	206	190	210	204	207	215	212	198
654	7	182	197	191	208	214	208	178	195
660	7	182	246	186	190	315	197	195	209
729	7	259	spill	288	276	285	280	279	271
Mean		244.0	239.7	239.8	256.6	266.5	253.7	242.5	249.0
Std Dev		72.9	62.5	55.0	68.6	78.5	73.8	73.2	67.5
SEM		18.8	16.7	14.7	17.7	20.3	19.0	18.9	17.4

Appendix J (cont.): WATER CONSUMPTION (ml/week)

Animal# 86D00-	Group	WK24	WK25	WK26	WK27	WK28	WK29	WK30
516	7	212	185	170				
518	7A	179	212	202	201	198	180	199
524	7A	302	275	258	231	233	221	229
535	7	222	221	198				
537	7A	229	230	238	223	443	237	248
547	7A	380	413	386	344	296	315	308
560	7	238	248	223				
569	7	172	170	172				
591	7A	297	272	277	255	317	251	252
597	7	418	411	450				
616	7	202	181	187				
623	7	198	189	204				
654	7	202	190	177				
660	7	200	186	174				
729	7	285	273	262				
Mean		249.1	243.7	238.5	250.8	297.4	240.8	247.2
Std Dev		73.2	77.0	81.5	55.6	94.4	49.3	39.9
SEM		18.9	19.9	21.0	24.8	42.2	22.0	17.9

Appendix K: BODY WEIGHTS (g)

Animal# 86D00-	Group	RPT[@]	ALC[$]	QWK1[†]	QWK2	WK1	WK2	WK3	WK4	WK5
517	1	121	178	182	238	289	329	357	390	412
532	1	127	186	191	255	303	336	374	399	428
538	1	106	163	173	228	282	319	361	381	412
540	1	129	93	92	183	258	309	346	384	415
558	1	101	142	152	202	255	294	334	362	395
576	1	116	180	185	250	307	347	385	415	439
578	1	121	125	164	227	284	302	367	398	426
579	1	123	148	181	252	308	355	398	428	459
584	1	109	76	102	165	229	279	322	358	394
585	1	115	160	166	218	265	298	331	347	366
614	1	122	187	196	260	331	380	428	468	507
647	1	115	81	104	172	239	299	343	384	419
665	1	114	175	178	238	296	335	364	400	420
715	1	112	167	172	230	275	300	331	358	546
725	1	109	168	173	232	291	336	373	398	415
Mean		116.0	148.6	160.7	223.3	280.8	321.2	360.9	391.3	430.2
Std Dev		7.9	37.8	33.6	30.0	27.7	27.4	28.5	30.8	44.9
SEM		2.0	9.8	8.7	7.8	7.2	7.1	7.4	7.9	11.6

[@] Receipt.
[$] Allocation.
[†] Quarantine week 1.

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK6	WK7	WK8	WK9	WK10	WK11	WK12	WK13	WK14
517	1	442	467	480	497	508	523	535	551	562
532	1	459	493	520	541	562	572	594	604	624
538	1	438	459	479	492	500	512	528	540	530
540	1	440	469	484	503	517	526	537	548	560
558	1	428	449	464	476	488	497	516	526	533
576	1	477	506	525	543	560	577	592	605	616
578	1	454	473	494	499	516	531	535	542	537
579	1	492	512	524	536	554	558	573	582	586
584	1	429	458	484	502	519	542	555	569	579
585	1	386	401	411	418	428	433	440	445	450
614	1	542	580	607	620	656	669	696	715	739
647	1	452	480	498	511	535	550	562	564	587
665	1	448	458	478	495	509	522	524	535	547
715	1	532	420	430	446	425	483	484	495	508
725	1	448	473	481	491	507	518	529	553	553
Mean		457.8	473.2	490.6	504.7	518.9	534.2	546.7	558.3	567.4
Std Dev		39.8	41.2	44.8	45.8	55.2	51.9	56.9	59.0	64.0
SEM		10.3	10.6	11.6	11.8	14.2	13.4	14.7	15.2	16.5

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK15	WK16	WK17	WK18	WK19	WK20	WK21	WK22
517	1	571	583	589	588	590	600	614	618
532	1	641	655	665	669	678	693	702	716
538	1	557	571	578	588	591	600	611	624
540	1	569	586	584	596	588	603	615	615
558	1	534	544	554	555	561	567	568	577
576	1	632	648	667	670	669	685	692	707
578	1	557	567	567	572	574	584	593	601
579	1	597	625	614	618	621	633	634	650
584	1	600	608	619	628	635	648	657	670
585	1	453	454	465	471	471	476	480	490
614	1	755	761	780	779	780	794	798	799
647	1	594	595	611	612	621	622	628	644
665	1	556	564	569	580	593	603	599	621
715	1	516	525	535	533	540	541	557	563
725	1	567	569	584	591	598	614	619	624
Mean		579.9	590.3	598.7	603.3	607.3	617.5	624.5	634.6
Std Dev		66.7	68.3	70.5	69.4	69.4	72.3	72.0	71.7
SEM		17.2	17.6	18.2	17.9	17.9	18.7	18.6	18.5

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK23	WK24	WK25	WK26	WK27	WK28	WK29	WK30
517	1	627	627	631	641				
532	1	716	728	729	746				
538	1	623	632	646	650				
540	1	610	626	641	651	646	651	655	655
558	1	553	583	593	601				
576	1	706	719	726	741				
578	1	606	610	622	626	622			
579	1	657	664	670	677	676			
584	1	672	671	686	687	696			
585	1	490	500	504	512	512	506	513	516
614	1	821	828	848	853	859	855	870	877
647	1	645	652	649	661	661	659	671	672
665	1	627	640	643	655	639	648	663	663
715	1	571	576	583	590	591			
725	1	625	633	633	640	646			
Mean		636.6	645.9	653.6	662.1	654.8	663.8	674.4	676.6
Std Dev		76.4	75.0	77.2	77.7	88.1	124.4	127.3	129.1
SEM		19.7	19.4	19.9	20.1	27.9	55.6	56.9	57.7

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	RPT[@]	ALC[$]	QWK1[†]	QWK2	WK1	WK2	WK3	WK4	WK5
503	2	136	98	99	192	241	307	345	379	402
515	2	123	176	187	248	325	376	427	472	502
549	2	113	81	80	156	217	274	312	351	381
582	2	112	170	179	251	296	340	379	406	428
594	2	131	189	203	268	320	369	407	444	473
610	2	103	113	117	185	237	270	268	328	349
653	2	118	92	96	175	248	304	338	384	412
693	2	104	166	174	234	282	316	348	380	407
710	2	141	206	211	263	343	378	420	447	481
732	2	104	83	97	167	234	283	318	363	396
Mean		118.5	137.4	144.3	213.9	274.3	321.7	356.2	395.4	423.1
Std Dev		13.8	48.4	50.9	43.0	44.7	41.8	51.5	46.2	48.2
SEM		4.4	15.3	16.1	13.6	14.1	13.2	16.3	14.6	15.2

[@] Receipt.
[$] Allocation.
[†] Quarantine week 1.

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK6	WK7	WK8	WK9	WK10	WK11	WK12	WK13	WK14
503	2	422	441	454	473	483	502	514	532	539
515	2	533	561	574	602	626	647	661	684	701
549	2	400	428	449	467	482	492	498	510	516
582	2	449	464	482	497	508	520	543	552	563
594	2	496	524	551	564	579	598	624	633	648
610	2	365	383	400	411	418	432	441	446	460
653	2	447	459	491	508	523	541	553	564	583
693	2	432	470	474	486	506	526	539	550	568
710	2	508	528	543	562	581	598	615	627	640
732	2	431	459	485	514	525	552	561	571	593
Mean		448.3	471.7	490.3	508.4	523.1	540.8	554.9	566.9	581.1
Std Dev		51.1	52.7	52.7	55.6	59.6	61.8	64.9	67.7	69.6
SEM		16.2	16.7	16.7	17.6	18.8	19.5	20.5	21.4	22.0

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK15	WK16	WK17	WK18	WK19	WK20	WK21	WK22
503	2	546	558	570	574	575	582	601	611
515	2	704	732	751	759	759	774	789	794
549	2	523	535	543	547	543	556	566	577
582	2	570	589	596	597	600	610	621	625
594	2	663	682	703	712	717	737	745	761
610	2	453	460	474	472	469	478	476	489
653	2	579	587	607	603	590	616	619	636
693	2	584	601	607	655	623	633	647	659
710	2	643	661	663	674	680	689	688	702
732	2	599	598	629	629	635	651	657	671
Mean		586.4	600.3	614.3	622.2	619.1	632.6	640.9	652.5
Std Dev		72.1	77.3	79.1	83.1	84.7	86.6	88.5	88.1
SEM		22.8	24.4	25.0	26.3	26.8	27.4	28.0	27.9

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK23	WK24	WK25	WK26	WK27	WK28	WK29	WK30
503	2	618	660	635	644	651			
515	2	796	808	808	832	841			
549	2	582	592	524	584	595			
582	2	629	631	639	636	643			
594	2	762	775	789	798	805			
610	2	496	503	515	512	515			
653	2	646	658	668	678	680			
693	2	673	677	688	686	692			
710	2	711	719	736	738	741			
732	2	683	692	701	694	702			
Mean		659.6	671.5	670.3	680.2	686.5			
Std Dev		87.0	87.4	98.0	95.0	95.5			
SEM		27.5	27.6	31.0	30.1	30.2			

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	RPT[@]	ALC[$]	QWK1[†]	QWK2	WK1	WK2	WK3	WK4	WK5
534	3	111	80	90	167	233	297	331	373	411
536	3	111	164	173	227	272	311	345	370	396
559	3A	127	168	169	230	290	335	377	405	430
571	3A	118	175	203	272	323	327	396	421	451
572	3	122	131	139	198	247	285	308	333	357
603	3	104	72	75	148	210	265	304	347	375
608	3A	124	176	186	245	284	310	337	362	375
613	3	105	159	164	220	267	309	338	371	403
622	3A	123	186	192	243	306	347	381	408	432
638	3	112	85	88	171	233	287	331	364	395
651	3A	106	158	165	224	280	318	354	388	424
658	3	102	99	103	172	226	269	309	345	376
662	3	123	149	152	228	287	333	372	410	445
671	3	106	169	174	234	274	308	346	375	405
701	3	107	168	171	239	293	337	377	409	436
Mean		113.4	142.6	149.6	214.5	268.3	309.2	347.1	378.7	407.4
Std Dev		8.6	39.0	41.1	35.2	32.1	24.8	28.7	27.0	28.6
SEM		2.2	10.1	10.6	9.1	8.3	6.4	7.4	7.0	7.4

[@] Receipt.
[$] Allocation.
[†] Quarantine week 1.

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK6	WK7	WK8	WK9	WK10	WK11	WK12	WK13	WK14
534	3	427	441	480	497	520	535	545	565	574
536	3	414	432	450	455	481	487	498	507	521
559	3A	455	462	486	499	512	528	538	548	552
571	3A	472	499	506	520	540	547	573	580	592
572	3	384	404	417	426	453	468	478	493	504
603	3	404	434	454	480	506	516	538	552	559
608	3A	393	405	414	418	432	437	452	457	454
613	3	425	450	469	482	503	516	529	544	558
622	3A	453	473	491	519	531	544	557	569	578
638	3	420	439	460	472	599	508	524	526	542
651	3A	445	470	488	515	522	533	539	561	568
658	3	404	419	442	459	476	480	487	497	509
662	3	474	495	521	545	563	586	594	612	589
671	3	427	452	470	489	510	527	543	562	576
701	3	464	485	505	515	534	501	561	564	581
Mean		430.7	450.7	470.2	486.1	512.1	514.2	530.4	542.5	550.5
Std Dev		28.6	30.0	31.3	35.7	41.6	36.3	37.7	39.7	38.4
SEM		7.4	7.7	8.1	9.2	10.7	9.4	9.7	10.3	9.9

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK15	WK16	WK17	WK18	WK19	WK20	WK21	WK22
534	3	591	600	611	622	630	639	647	663
536	3	537	531	546	549	552	564	572	580
559	3A	573	575	574	585	594	604	608	618
571	3A	598	611	619	632	637	636	646	659
572	3	515	522	527	537	544	553	556	568
603	3	562	588	585	595	602	610	620	631
608	3A	453	467	486	489	477	475	488	492
613	3	567	572	578	587	589	593	605	613
622	3A	590	602	620	631	631	636	655	664
638	3	559	565	579	581	596	605	617	625
651	3A	581	585	600	599	607	623	635	638
658	3	514	522	536	547	546	559	568	567
662	3	626	645	652	667	663	672	686	698
671	3	593	606	615	628	626	647	653	664
701	3	594	612	625	630	638	647	655	666
Mean		563.5	573.5	583.5	591.9	595.5	604.2	614.1	623.1
Std Dev		43.5	45.9	44.3	46.4	48.3	50.0	50.6	53.0
SEM		11.2	11.8	11.5	12.0	12.5	12.9	13.1	13.7

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK23	WK24	WK25	WK26	WK27	WK28	WK29	WK30
534	3	664	660	669	668	682			
536	3	593	594	597	598	605			
559	3A	613	633	643	646	658	653	658	659
571	3A	666	673	687	692	684	688	696	695
572	3	556	572	577	573	590			
603	3	620	633	653	633	641			
608	3A	505	509	514	511	523	525	522	530
613	3	625	630	640	627	632			
622	3A	672	673	675	688	685	687	686	692
638	3	639	641	653	654	663			
651	3A	639	652	657	668	669	672	672	670
658	3	581	575	587	591	602			
662	3	712	715	730	734	741			
671	3	668	681	689	696	710			
701	3	672	674	695	689	708			
Mean		628.3	634.3	644.4	644.5	652.9	645.0	646.8	649.2
Std Dev		53.2	52.8	55.2	57.6	55.8	68.6	71.2	68.3
SEM		13.7	13.6	14.3	14.9	14.4	30.7	31.9	30.6

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	RPT[@]	ALC[$]	QWK1[†]	QWK2	WK1	WK2	WK3	WK4	WK5
516	7	117	174	179	247	281	329	353	371	398
518	7A	112	173	183	244	289	358	397	426	455
524	7A	110	157	163	219	252	300	331	363	389
535	7	135	191	203	255	293	346	374	398	419
537	7A	128	93	101	186	230	296	325	349	372
547	7A	113	80	168	231	261	318	348	381	400
560	7	108	171	179	249	285	347	383	416	453
569	7	101	111	149	206	237	287	307	334	359
591	7A	137	98	118	207	260	339	374	416	450
597	7	119	84	88	141	185	260	295	332	369
616	7	142	189	197	259	295	361	395	432	451
623	7	123	183	192	251	285	344	375	405	434
654	7	117	133	133	204	246	310	348	384	414
660	7	124	92	115	186	229	291	329	358	387
729	7	110	170	170	227	263	322	356	381	409
Mean		119.7	139.9	155.9	220.8	259.4	320.5	352.7	383.1	410.6
Std Dev		11.7	42.4	36.6	32.9	30.6	29.5	30.9	32.2	32.5
SEM		3.0	10.9	9.4	8.5	7.9	7.6	8.0	8.3	8.4

[@] Receipt.
[$] Allocation.
[†] Quarantine week 1.

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK6	WK7	WK8	WK9	WK10	WK11	WK12	WK13	WK14
516	7	427	439	451	459	478	482	494	504	511
518	7A	475	501	521	537	553	558	575	578	598
524	7A	402	429	452	466	486	505	504	516	528
535	7	433	463	486	500	517	529	541	554	569
537	7A	392	416	432	444	458	474	483	488	507
547	7A	427	455	474	480	488	498	505	525	537
560	7	482	518	540	545	566	587	605	622	641
569	7	380	404	423	436	450	461	473	473	474
591	7A	473	504	523	539	556	571	590	604	626
597	7	402	403	408	417	480	504	484	529	547
616	7	486	509	521	531	552	563	580	596	611
623	7	465	492	510	513	544	547	564	575	608
654	7	449	469	491	511	522	517	552	562	569
660	7	415	438	463	481	507	516	529	535	556
729	7	4[illegible]	447	468	486	506	507	513	536	553
Mean		435.9	459.1	477.5	489.7	510.9	521.3	532.8	546.5	562.3
Std Dev		34.4	38.6	40.2	40.1	37.3	37.2	43.0	43.2	47.6
SEM		8.9	10.0	10.4	10.3	9.6	9.6	11.1	11.2	12.3

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK15	WK16	WK17	WK18	WK19	WK20	WK21	WK22
516	7	509	519	526	539	533	542	553	553
518	7A	611	627	630	638	648	660	671	676
524	7A	533	539	536	546	563	570	574	576
535	7	582	596	598	610	613	624	629	634
537	7A	508	521	521	530	534	544	551	554
547	7A	534	546	544	558	555	561	562	560
560	7	657	668	681	693	694	702	706	712
569	7	487	491	504	505	509	514	522	527
591	7A	625	649	656	659	668	677	681	685
597	7	504	525	521	555	546	571	549	584
616	7	614	625	646	650	653	671	673	682
623	7	613	627	633	637	638	639	645	647
654	7	572	581	594	595	600	610	615	624
660	7	566	572	587	584	594	622	612	619
729	7	561	585	592	599	605	609	617	622
Mean		565.1	578.1	584.6	593.2	596.9	607.7	610.7	617.0
Std Dev		51.7	53.6	56.6	54.4	55.8	56.1	56.9	56.6
SEM		13.3	13.8	14.6	14.1	14.4	14.5	14.7	14.6

Appendix K (cont.): BODY WEIGHTS (g)

Animal# 86D00-	Group	WK23	WK24	WK25	WK26	WK27	WK28	WK29	WK30
516	7	560	567	573	574				
518	7A	687	648	680	684	687	692	699	698
524	7A	582	592	600	597	598	598	600	598
535	7	644	651	666	662				
537	7A	568	571	583	585	586	508	566	577
547	7A	570	570	580	585	573	573	578	584
560	7	721	727	745	752				
569	7	529	533	537	533				
591	7A	699	708	718	722	725	738	735	731
597	7	586	588	591	590				
616	7	692	691	702	704				
623	7	655	647	658	676				
654	7	632	637	648	647				
660	7	628	636	645	647				
729	7	634	635	641	647				
Mean		625.8	626.7	637.8	640.3	633.8	621.8	635.6	637.6
Std Dev		58.2	56.0	59.5	61.8	67.8	92.6	76.4	71.6
SEM		15.0	14.5	15.4	16.0	30.3	41.4	34.2	32.0